VOLATILE PHYTOTOXINS IN FOREST LITTER

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ABSTRACT

Volatile inhibitors in *Pinus radiata* litter, which may affect the establishment of second generation *P. radiata* or pasture growth beneath *P. radiata* trees, were investigated. A bioassay technique was devised to ascertain the effects of vapour from incubated litter samples. Litter vapour caused growth inhibition of clover (*Trifolium repens*), ryegrass (*Lolium perenne*) and pine (*P. radiata*) seedlings. The inhibitor was identified as ethylene by using absorbent and cold traps and confirmed by gas chromatographic analysis. Litter vapour also inhibited seed germination although in some instances clover germination was stimulated. It was found that ethylene and carbon dioxide were responsible for these effects. Under laboratory conditions, litter was shown to produce ethylene but ethylene was not detected in vapour samples from the field. Volatile compounds from *P. radiata* litter are not thought to affect plant growth under normal field conditions.

CHAPTER I

INTRODUCTION

I. INTRODUCTION AND AIMS

Pinus radiata D. Don was introduced to New Zealand towards the end of the nineteenth century and was found to grow exceptionally well. In the 1920s the newly formed Forest Service began exploiting this potential and rapidly planted large areas to provide a commercial substitute for the slower growing indigenous species. *P. radiata* forests now provide a majority of the timber felled in N.Z. (Scott, 1960).

Although *P. radiata* has been widely researched there is no mention in the literature of allelopathic relationships with undergrowth species or with *P. radiata* seedlings. Allelopathy refers to deleterious interactions between plants (both inter- and intra-specific) brought about by chemicals in leaf or root exudates or from the decomposition of plant material.

Interest in allelopathy stems from a problem encountered in Nelson, N.Z., where second generation *P. radiata* has been shown to grow more slowly than the first crop (Whyte, 1973). The land where this problem occurs is infertile, but though extensive fertiliser trials have shown a number of deficiencies (Will *et al.*, 1963; Stone and Will, 1965; Appleton and Slow, 1966; Adams, 1971) they have not fully accounted for the reduced growth. One explanation is that chemicals released during decomposition of residues of the first crop cause growth inhibition of the second crop. Although this is the only documented case in N.Z. it has

been described for Australian P. radiata forests (Keeves, 1966; Bednall, 1968; R. Boardman, pers. comm.).

Another situation in which knowledge of the allelopathic potential of P. radiata could be applied is the management system integrating forestry with pastoral farming in which an open stand of P. radiata is maintained and the grass sward utilised for hay making and grazing with sheep and cattle. This provides an immediate return in the form of agricultural products and allows maximum tree growth by freeing them from intraspecific competition (Tustin and Knowles, 1975). A study of effects the trees have on the pasture plants may enable improved management techniques to provide higher grass yields.

In the P. radiata forests of Canterbury, N.Z., there is a paucity of undergrowth which is usually attributed to competition for moisture and to low light intensity, although the situation also occurs under open, high pruned stands. Moist conditions in the forests during spring are favourable for seed germination but few seedlings, including P. radiata, establish. Inhibition of seed germination and seedling establishment may be caused by compounds from the forest litter as has been shown in Juniperus osteosperma - P. edulis forests in Arizona by Jameson (1970).

The aim of this project was to establish whether or not litter from P. radiata forests in Canterbury, N.Z., produced volatile substances inhibitory to seed germination and seedling growth. Identification of any inhibitory compounds would be followed by an investigation into the conditions under which they are produced and whether or not they occur in the field at inhibitory concentrations. This complements research on water soluble toxins in progress at the Forest Research Institute,

Rotorua.

II. LITERATURE

(1) Studies on allelopathy

The subject of allelopathy has been well covered for all plants in several reviews (Bonner,1950; Borner, 1960; Patrick et al., 1964; Muller C.H., 1966; Florence, 1967; Rice, 1967; De Bell, 1970; Patrick, 1971; National Academy of Science, 1971) but only studies on trees involved in allelopathic relationships will be mentioned here.

In a survey of the allelopathic potential of forty dominant plant species in Washington State, U.S.A., Del Moral and Cates (1971) found that nine species showed strong inhibition in laboratory assays and reduced understory growth in the field. Their findings suggest that allelopathic interactions may be common in plant communities. Brown (1967) investigated the toxicity of leaf extracts of fifty six plant species found in Pinus banksiana stands and found that nine species consistently inhibited seed germination and seedling growth of P. banksiana. He concluded that biologically active chemicals influenced plant succession and distribution. Leaf extracts of Quercus falcata were implicated in the retardation of understory growth beneath these trees (De Bell, 1971) hence affecting the distribution of plants in the community. Brown (1967) used leaf extracts in his toxicity tests and therefore the results may not be valid in a field situation.

Leaves are homogenised before extraction so compounds may be present in the extract which are not normally exuded from the leaf. Washings of leaves and roots contain a sample of the chemicals being released into the environment by the plant and

therefore give a more realistic indication of the influence of exudates on other plant species. The exclusion of undergrowth from stands of Juglans nigra has been attributed to the presence of the toxin juglone in leaf washings from the trees (Bode, 1958). Toxins in the fog drip from Eucalyptus globulus have been suggested as a cause of the lack of undergrowth beneath these trees (Del Moral and Muller, 1969). Al-Naib and Rice (1971) found toxic compounds in leaf washings of Platanus occidentalis and thought that these as well as products from decaying leaves caused reduced herb growth beneath the canopy. All these examples indicate that leaf exudates washed to the soil by rain may cause growth inhibition of understory plants.

Root exudation is a well known phenomenon and washings of tree roots have been shown in some cases to be toxic. Bevege (1968) showed that root washings of Araucaria cunninghamii, Pinus elliottii and Flindersia australis were toxic to seedlings of A. cunninghamii with the intraspecific factor being less active than the interspecific factors. A water-transferable component from the roots of Grevillea robusta was shown to be toxic to G. robusta seedlings (Webb et al., 1967) and the authors attributed the non-gregarious habit of the tree to this auto-toxicity. Titov (1971) showed that isolating grasses from spruce roots by trenching had a greater effect on grass growth than fertilising and he proposed that exudates from the spruce roots reduced grass growth.

Decomposition products of plant residues have also been studied in this context. In the replant disease of peach the breakdown products of a cyanophoric glucoside, amygdalin, have been associated with the very poor growth of young peach trees planted on old orchard land (Patrick, 1955). Amygdalin is

present in the roots of old peach trees and yields the toxin benzaldehyde on decomposition by micro-organisms. Jameson (1970) detected an active growth inhibitor which accumulated in the humus beneath Juniperus osteosperma which he thought caused the reduced grass growth beneath the canopy. These two studies on residues from trees indicate that the breakdown of plant parts either in litter or in the soil can yield substances detrimental to plant growth.

Products from litter could also affect the distribution and growth of micro-organisms. Smith R.S. (1967) investigated the absence of Fusarium oxysporum from Pinus lambertiana forests and found that four years after planting out P. lambertiana seedlings from a contaminated nursery no F. oxysporum could be isolated from the root zone of the transplanted seedlings. He concluded that some environmental pressure within the forest soil eventually eliminated introduced populations of F. oxysporum. Hammerschlag and Linderman (1974) showed that five acids from pine needles stimulated germination of F. oxysporum spores and then caused lysis of the germ tube and they suggested this as a mechanism by which F. oxysporum is excluded from conifer forest soils. Micro-organisms involved in litter breakdown may also be affected by chemicals from the litter. Knowles and Laishley (1959) attributed the slow decomposition of Fagus grandifolia litter to fungistatic compounds from fallen leaves and Kowal (1969) showed that leaching Pinus echinata litter increased its rate of decomposition, probably by removing fungistatic substances.

Not only may the activity of micro-organisms be affected but also the balance of species necessary for favourable tree growth. Florence (1965) studied the decline in growth of old redwood forests and their failure to regenerate and he decided

that the problem was caused by a deterioration in the microbial processes of nutrient cycling and in some cases to an increase in the numbers of root pathogens brought about by the long term addition of redwood litter to the soil. In an investigation into the poor regeneration of Abies alba, Maliszewska and Moreau (1960) proposed that accumulations of plant excretions or decomposition products caused either a direct toxic action on young trees or an upset in the desirable microbial balance. Florence and Crocker (1962) demonstrated a microbial environment in soil from an Eucalyptus pilularis forest which was severely antagonistic to E. pilularis seedlings. They suggested that a specialised microbial complex developed in the forest which provided favourable incorporation of organic matter into soil humus but which was unfavourable to plant growth. These workers concluded that plant products and their effects on micro-organisms play an important part in the establishment of plant community relationships.

Allelopathy appears to be a widespread phenomenon with important implications in the distribution of plants within a community. In some instances these interactions may be of economic importance, particularly in forestry.

(2) Effects of forest litter

Some field situations described by foresters could be interpreted as allelopathic relationships. Person and Hallin (1942) found that restocking of cutover redwood was greatly enhanced by burning the slash because this removed the unfavourable effect of the undisturbed litter layer. In a paper on restocking spruce in a mixed hardwood stand, Koroleff (1954) considered that leaf litter played a major role in preventing seedling establishment by providing a mechanical barrier which 'smothered'

the seedlings before they reached the light. Grano (1949) reported an inverse relationship between litter depth and seedling establishment in Pinus taeda - P. echinata forests. The growth of grasses and legumes beneath stands of P. palustris and P. elliottii was greatly enhanced by removal of the litter layer, but was again reduced by subsequent needle cast (Halls and Suman, 1954). Although Koroleff's (1954) interpretation may be correct, established plants can also be affected by needle litter. Toxic compounds released from the litter could be causing these effects.

(3) Volatile substances inhibitory to plant growth

Many volatile compounds have been shown to cause inhibition of germination and growth of higher plants. Ethanol, as a fungal metabolite in association with carbon dioxide, can cause chlorosis and reduced elongation of lettuce seedlings (Hutchinson and Cowan, 1972). Ethanol, acetaldehyde and acetone have been shown to inhibit weed seed germination, particularly at reduced oxygen tensions (Holm, 1972). Butyric acid and ammonia were the main components toxic to the seeds and sprouts of wheat in a study by Samtesvich and Borisova (1963). Toxic concentrations of hydrogen cyanide from microbial cultures have been demonstrated (Marshall and Hutchinson, 1970). Poor growth of trees in heath soils at Wareham Forest, England, was attributed to hydrogen sulphide formed by sulphur reducing bacteria in anaerobic conditions by Neilson-Jones (1941) who also showed the presence of a volatile factor in the soil which caused an epinastic response in tomato seedlings similar to the response caused by ethylene. Both higher plants (Abeles, 1973) and micro-organisms (Ilag and Curtis, 1968; Hutchinson, 1973) produce ethylene which, at low concentrations;

usually inhibits seedling growth (Abeles, 1973). Plhak and Urbankova (1969) showed that cereal roots produced ethylene which, they suggested, might influence root elongation of associated plants. Smith K.A. and Restall (1971) showed the presence of sufficient ethylene in anaerobic soils to restrict the root growth of cereals.

There are instances of more unusual volatile compounds causing germination and growth inhibition of plants. Sprouting of potato tubers was inhibited by three aromatic substances (benthiazole, 1,4-dimethylnapthalene and 1,6-dimethylnapthalene) produced by stored tubers (Meigh et al., 1973). Hexa-1,3,5-triyne, a product from cultures of Fomes annosus, caused complete inhibition of germination of cress seed (Glen and Hutchinson, 1973). Monoterpenes produced by Salvia leucophylla reduced cell elongation and mitosis in Cucumis sativus seedlings (Muller W.H., 1965).

This literature shows the range of volatile compounds which can affect plant germination and growth.

CHAPTER II

MATERIALS AND METHODS

I. SAMPLING PROCEDURE

Litter samples were collected from a mature Pinus radiata stand at Ashley Forest, Canterbury. The trees were 39 years old and stocked at 300 stems Ha^{-1}. Samples were taken from the south facing upper slope of a secondary ridge where the undergrowth was sparse and the litter depth was 6.4 cm (S.E. 3.9 cm). Details of climate and soil type are given in Appendix 1.

Samples were selected using random co-ordinates within a 100 m^2 plot and were placed in 18 litre galvanised iron containers. * 1
Initially, five samples were taken for each container but to reduce variability this was increased to twenty. A 10 cm diameter steel corer was made to ease the cutting of samples and in subsequent experiments it was used to take 40 litter cores for each container. The complete litter horizon to the surface of the mineral soil was sampled. * 2

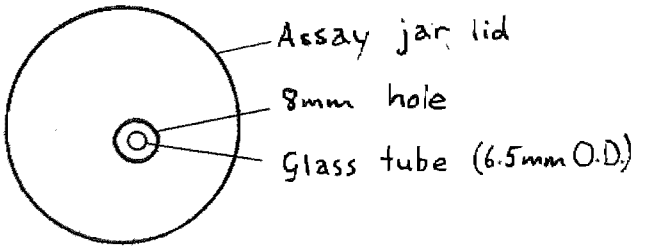
II. TREATMENT OF LITTER PRIOR TO BIOASSAY

In preliminary experiments a litter sample was placed in a sealed container with the seedlings used for assaying the effects of litter vapour on growth. Results from these * 3
experiments were variable because the litter samples used were small, so a system was devised in which a larger composite litter sample was placed in an 18 litre container fitted with inlet and

and outlet tubes (Fig. 1). To maintain the humidity within the litter container, 200 ml distilled H_2O in a 500 ml beaker was included and the container sealed. After incubation at 25°C for four days the atmosphere within the container was displaced by compressed air and conducted to the assay material through glass tubing. A flow rate of 0.8 litre min^{-1} was maintained using a water manometer and a constriction in the glass tube. The assay material was placed on moist Whatman Seed Test paper in 500 ml Agee jars and one litre of vapour was flushed over it through a hole in the metal seal. The hole was then sealed and the jars incubated at 25°C for the requisite time. In some experiments the litter containers were resealed and incubated for a further seven days. Controls of empty litter containers were treated identically to those containing litter. * 4

In some experiments litter vapour was passed through absorbent traps (Fig. 1). A paraffin wax trap was prepared by packing wax shavings into a gas washing bottle. The absorbent used for the potassium permanganate trap was prepared by immersing 100 g silica gel in 125 ml 0.1 M $KMnO_4$ and drying overnight at 105°C (Abeles, 1973). This material was then placed in a gas washing bottle. The cold trap used was as described by Dal Nogare and Juvet (1965, p253). The two coolants were liquid nitrogen (-195°C) and diethyl ether - dry ice (-100°C). * 5 Anhydrous $MgSO_4$ was used as a drying agent.

Assay jars were normally treated consecutively, but in some germination experiments two manifolds, each with six outlets, were used. These were connected as shown in Fig. 2. When an absorbent trap was used this was placed as indicated and the flow through the other manifold balanced by using a valve. Flow rates were measured with bubble meters before the manifolds were attached.



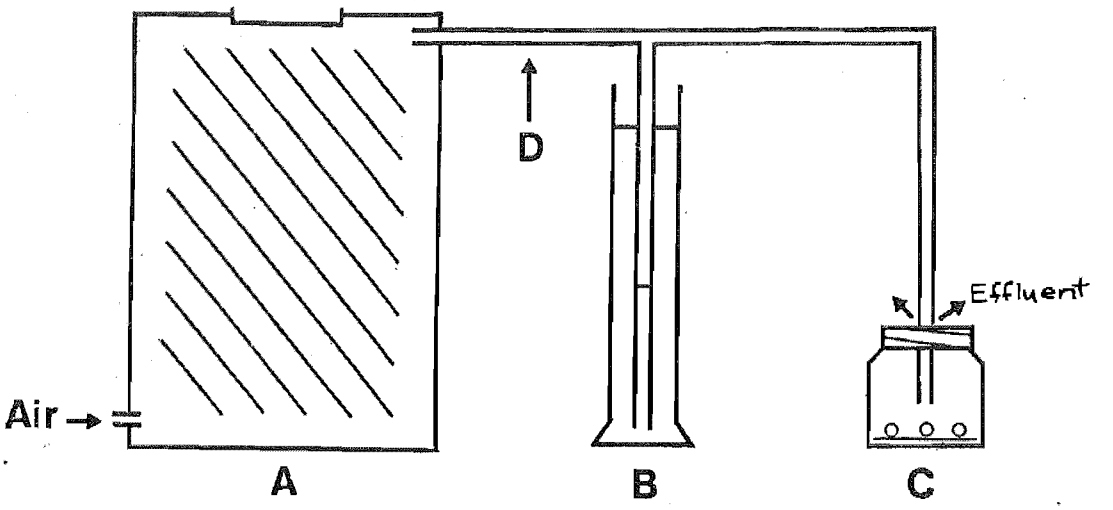


Fig. 1 Apparatus used for assaying the effects of vapour from *P. radiata* litter.

A – litter container (14 cm x 24 cm x 34 cm)

B – water manometer

C – assay jar

D – position of absorbent trap

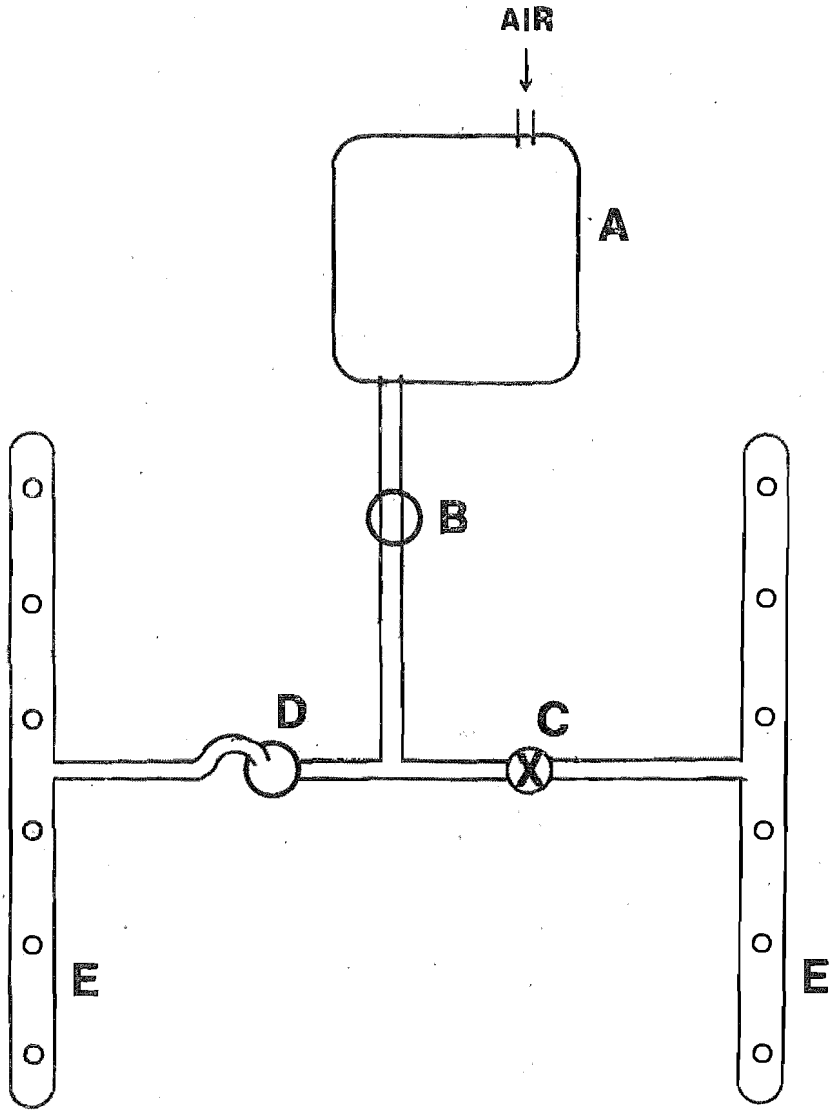


Fig. 2 Plan of modified appamatus used for assaying the effects of P. radiata litter vapour on seed germination.

A - litter container

B - water manometer

C - valve

D - absorbent trap

E - manifold

III. PLANT MATERIAL FOR BIOASSAY

In preliminary experiments, lettuce (Lactuca sativa L., 'Imperial Summer' supplied by Arthur Yates and Co. Ltd.) and radish (Raphanus sativus L., 'French Breakfast' supplied by F. Cooper Ltd.) seedlings were used. Seeds were surface sterilised for five minutes in 14% (v/v) Janola (3.5% NaOCl), rinsed in sterile water and germinated on moist sterile filter paper in 9 cm Petri dishes for 24 h at 25°C. Seedlings with uniform appearance were selected for assay, and radicle length measured after two days in the presence of litter.

Grasslands 'Huia' white clover (Trifolium repens L.) and Grasslands 'Ruanui' ryegrass (Lolium perenne L.) seed was supplied by Grasslands Division, Department of Scientific and Industrial Research, Lincoln, N.Z.. Surface sterilisation procedure was as described above. Clover seed was germinated on filter paper in 23 cm diameter Petri dishes for 24 h at 25°C before selecting seedlings for assay. Hypocotyl growth was measured to the nearest millimetre after four days incubation at 25°C following treatment with vapour.

Ryegrass seed was germinated for 48 h before selecting seedlings for assay. Plumule growth, from the point of emergence from the seed to the shoot tip, was measured four days after treatment with vapour.

Pine (Pinus radiata D. Don) seed was supplied by the Forest Research Institute, Rotorua. It was washed in running water for 24 h, stratified at 4°C for 24 h and surface sterilised with 30% (w/w) H_2O_2 for 30 minutes. Hydrogen peroxide increases the germination rate (Trappe, 1961) which is usually slow and variable. The seed was germinated for five days in sterile

vermiculite before transferring selected seedlings to damp filter paper in assay jars. Hypocotyl growth was measured after seven days incubation following treatment with vapour.

'Tan-ginbozu' rice (Oryza sativa L.) seed was surface sterilised with Janola as described above and germinated for 60 h before selecting seedlings for assay. Plumule length was measured five days after treatment with vapour.

Usually 15 seedlings were placed in each assay jar and five jars treated for each litter container.

In germination tests, dry clover or ryegrass seed was placed on moist filter paper in the assay jars just prior to treatment with vapour. Pine seed was pretreated as described above and placed on filter pater in the assay jars immediately after the H_2O_2 soak. Normally 100 or 50 seeds were placed in each assay jar. A seed was classed as germinated if the radicle protruded through the seed coat.

IV. ANALYSIS OF DATA

Variances between treatments in the experiments of seedling growth were generally non-homogeneous, so to correct this for analysis of variance a logarithmic transformation was performed on the data before analysis. However, for ease of interpretation, the means presented in the tables of results are back-transformed (B-T). The experiments generally have a nested design, or in some cases a combination of nested and factorial. Where appropriate, results of F tests will be presented. To aid interpretation of the results the treatment means within an experiment were compared using the Student-Newman-Keul test (S.N.K.) for a posteriori testing (Sokal and Rohlf, 1969, p 239). Results of this test are

given in the tables of back-transformed means, although the test was made on the means of the transformed data. Means with the same superscript are not significantly different at the 5% level. The coefficients of variation (C.V.) given in the tables are of the transformed data.

Germination percentages frequently approached either 0% or 100% so to correct for the resultant skewed distribution an angular transformation of the data was used. Means presented in the tables are back-transformed and significant differences (5% level) are indicated by superscripts.

V. GAS CHROMATOGRAPHY

Analyses of vapour were carried out on a Tracor 550 gas chromatograph (G.C.) using a flame ionisation detector. Initially a 1.75 m x 3.2 mm copper column packed with 80-100 mesh Porapak Q (Column A) was used with a nitrogen flow rate of 60 ml min^{-1} and an oven temperature of 65°C. This column effected poor separation of ethylene and acetylene so in certain experiments a 3.05 m x 3.2 mm copper column packed with 100 mesh deactivated alumina (Smith K.A. and Dowdell, 1973) with a nitrogen flow of 30 ml min^{-1} and an oven temperature of 120°C (Column B) was used. For higher sensitivity the same packing was used in a 1.22 m x 3.2 mm column with a nitrogen flow of 60 ml min^{-1} and an oven temperature of 90°C (Column C). In all cases the detector and injection port temperatures were 270°C and 120°C respectively.

Ethylene and acetylene standards were dilutions of commercial gas from N.Z. Industrial Gases Ltd.. Ethane and propane were from Matheson Ltd., Australia. The concentrations were calculated from the standards using the mean peak height of two injections.

Samples (1 ml) were injected using a Hamilton gas tight syringe. Injecting 2 ml samples with plastic disposable syringes was satisfactory and the larger sample size increased the sensitivity. These syringes were also used for sampling vapour in the field.

CHAPTER III

RESULTS

I. THE EFFECTS OF LITTER VAPOUR ON SEEDLING GROWTH

In this section results are given of work establishing the technique and investigating the capacity of Pinus radiata litter to produce volatiles capable of affecting seedling growth.

(1) Determination of the technique

Experiments were carried out using a technique similar to that described by Persidsky and Wilde (1954) and Muller W.H. (1965) in which the source material, in this case litter, and the assay seedlings were enclosed together. Results of six experiments are given in Table 1. Two experiments using lettuce showed very high variation, and because of this the reduction in growth was not statistically significant. The level of inhibition in different experiments ranged from 37.3% to 79.2% of the control. This inconsistency appeared to be caused by non-uniformity in the litter samples, so a larger composite litter sample was used to provide vapour for assay. Lettuce and radish seedlings were used in these preliminary experiments but in subsequent work clover, ryegrass and pine were used because of their greater relevance to the field situation.

Radicle growth in some of the preliminary experiments showed high variation, so a comparison was made between clover radicle and hypocotyl growth. Three different conditions of moisture were also tested to provide information for subsequent

Table 1 Radicle growth of lettuce and radish seedlings in the presence of vapour from litter of P. radiata (B–T means in mm).

Expt.	Species	Control	Litter	% control	Sign.*	C.V.(%)
1	Lettuce	11.08	5.98	54.0	N.S.	45
2	Lettuce	8.89	6.18	69.5	***	17
3	Lettuce	10.63	8.42	79.2	***	8
4	Lettuce	15.61	5.82	37.3	N.S.	109
5	Radish	34.85	32.06	92.0	N.S.	7
6	Radish	24.15	25.96	107.5	N.S.	14

* Significance level of F tests; N.S. = not significant, *** = 0.1% level.

assay work. Ten germinated clover seedlings were placed in each of ten Petri dishes for each moisture level. After three days incubation at 25°C radicle and hypocotyl growth were measured (Table 2). In all cases hypocotyl growth showed a lower coefficient of variation than radicle growth and the difference was particularly marked at the highest moisture level. Hypocotyls also grew more than radicles, giving a larger range for demonstration of inhibitory effects. Hypocotyl growth was used in subsequent bioassays.

Table 2 The effect of moisture level on clover radicle and hypocotyl growth after three days at 25°C.

Water (ml)	Parameter measured	Mean (mm)	C.V. (%)
4	Hypocotyl	10.70	23
	Radicle	13.90	45
6	Hypocotyl	16.31	23
	Radicle	12.18	26
10	Hypocotyl	23.14	13
	Radicle	9.14	29

The technique using litter containers and assay jars was tested without litter to assess the variation inherent in the technique. Vapour from eight empty litter containers was assayed with clover seedlings. Although the second level of nesting gave a significant F test over the lowest level (Table 3), there was no difference between the containers. Attempts to reduce the variation between jars were unsuccessful and throughout the study this variance was significant when tested over the variance within jars. So the variance between jars was used in F tests on treatment variances and in S.N.K. tests.

Table 3 Analysis of variance of clover hypocotyl growth. Vapour from eight empty containers was assayed using five jars of 25 seedlings for each container.

Source of variation	Degrees of freedom	Mean square	F ratio	Sign. level*
Between containers	7	26.47	0.7	N.S.
Between jars within containers	32	40.28	4.7	***
Within jars	960	8.64		

* N.S. = not significant, *** = 0.1% level.

(2) Responses of clover, ryegrass and pine seedlings to litter vapour

In an initial experiment using five 0.1 m^2 litter samples for each container, vapour from three litter containers and one control was assayed with clover seedlings. The results (Table 4) show that vapour from each litter container had a different effect, with vapour from two litter containers causing significant inhibition.

In a fresh collection of litter, 20 samples were taken for each container and, after four days' incubation, vapour from six litter containers and two controls was assayed using clover and

Table 4 The effect of vapour from P. radiata litter on clover hypocotyl growth (B-T means in mm). Ten jars of 20 seedlings were treated for each container. (Superscripts indicate differences at the 5% level)

	Control	Litter vapour		
		1	2	3
Growth (mm)	15.70^{a}	15.55^{a}	7.96^{c}	12.34^{b}

C.V. = 22%

ryegrass seedlings. After incubation of the litter for a further seven days the assay was repeated using only clover seedlings. Because the results were consistent between containers, pooled means for controls and litter containers are given (Table 5). Although there is an absolute difference in clover growth between the assays of vapour from litter incubated for four and eleven days, the level of inhibition when expressed as a percentage of control is similar (36% and 39%). The difference in growth may have been caused by slight fluctuations in incubation conditions. Ryegrass seedling growth was also inhibited by litter vapour (74% of control) but appeared to be affected less than clover growth.

Table 5 The effect of litter vapour on the growth of ryegrass and clover seedlings (B-T means in mm). Five jars of 15 seedlings were treated for each container. (Superscripts indicate differences at the 5% level)

Time (days) of litter incubation	Species	Treatment	
		Control	Litter vapour
4	Ryegrass	27.76^{a}	20.58^{b}
4	Clover	17.61^{c}	6.40^{e}
11	Clover	15.24^{d}	5.94^{f}

C.V. = 23%

To check these results with another collection of litter two groups of containers were set up, each having three litter containers and one control. After four days litter incubation vapour from one group was assayed with clover and ryegrass seedlings. and from the other with clover and pine seedlings. Five jars of 15 seedling of each species were treated for each container. Significant inhibition of growth (Table 6) occurred for all species, with the levels of inhibition for clover and ryegrass being similar to those shown in the preceding experiment (32% and 68% respectively). Inhibition of pine seedlings (61%) was similar to that of ryegrass.

Table 6 The effect of vapour from P. radiata litter on the growth of ryegrass, clover and pine seedlings (B-T means in mm, Superscripts indicate differences at the 5% level.).

Species	Treatment	
	Control	Litter vapour
Ryegrass	32.05^{a}	21.71^{b}
Clover	18.18^{b}	5.86^{d}
Pine	21.96^{b}	13.36^{c}

C.V. = 28%

(3) The trapping effect of paraffin wax and water

Absorbent traps were used to provide more information on the chemical nature of the inhibitor. Water was selected as an absorbent to check on possible toxic concentrations of water soluble compounds, especially volatile metabolites such as ethanol, acetic acid, acetaldehyde, acetone and butyric acid. Vapour from two litter containers and one control was assayed with clover to compare untreated vapour with vapour which had passed through the water trap. Five jars of 15 clover seedlings were used per

container for each treatment.

Vapour from a second group of containers was passed through a wax trap. Monoterpenes produced by Salvia shrubs are reported to dissolve readily in paraffin wax (Muller C.H., 1965) and as terpenes are a component of pine resin, their possible involvement in the inhibitory effect was checked in this way. The effectiveness of the trap was qualitatively assessed by allowing 3 mg camphor to evaporate in a five litre flask and then passing the vapour through the trap. The characteristic odour of camphor was not detected by three impartial observers, but was distinctive in the untreated vapour.

Both traps increased the inhibitory effect (Table 7) and possible reasons for this will be discussed later. As the traps did not reduce the inhibition it seems that the inhibitor is not readily soluble in water or paraffin wax.

Table 7 The effect of water and wax traps on the inhibitory effect of litter vapour on clover growth (B-T means in mm. Superscripts indicate differences at the 5% level.).

Treatment of vapour	Container contents	
	Control	Litter
Untreated	19.04^{a}	7.14^{b}
Water trap	18.44^{a}	6.47^{c}
Wax trap	19.27^{a}	6.55^{c}

C.V. = 18%

(4) The effects of accumulated CO_2 and depleted O_2

Respiratory activity in the sealed litter containers would result in an accumulation of CO_2 and a depletion of O_2 over the incubation period. The effect of this on seedling growth was checked by passing vapour from two litter containers and one control

through a trap containing 0.9 N NaOH before assaying with five jars of 15 clover seedling per container for each treatment. The NaOH was titrated and minimum CO_2 concentrations calculated. These were 5.5% and 0.5% for litter vapour and control vapour respectively. Removal of CO_2 caused a slight increase in inhibition (Table 8) indicating that it is probably not responsible for the inhibition.

Table 8 The effect of removing CO_2 on the response of clover hypocotyl growth to litter vapour (B-T means in mm. Superscripts indicate differences at the 5% level.).

Treatment of vapour	Container contents	
	Control	Litter
Untreated	19.26^a	7.52^b
NaOH trap	19.52^a	5.51^c

C.V. = 22%

With a second group of containers, 200 ml 1.5 N NaOH was included instead of water to trap CO_2 produced within the containers during the incubation period. It was intended to replace the trapped CO_2 with O_2 according to the pressure change indicated by a manometer, but no change occurred so O_2 was not added. The size of the container may have prevented efficient trapping. To check the effect of O_2 depletion ten assay jars, each containing 15 clover seedlings, were treated for each container and 50 ml O_2 injected into alternate jars. Nitrogen (50 ml) was added to the remaining jars as a control. No difference occurred between the treatments (Table 9) suggesting that O_2 depletion was not responsible for the inhibitory effect.

Table 9 The response of clover hypocotyl growth to litter vapour after addition of O_2 (B-T means in mm. Superscripts indicate differences at the 5% level.).

	Container contents	
	Control	Litter
50 ml O_2 per jar	19.00^a	5.92^b
50 ml N_2 per jar	20.63^a	6.53^b

C.V. = 14%

(5) The effect of $KMnO_4$ and cold traps

These traps were used to check the possibility that ethylene (C_2H_4) might be causing the inhibition. Ethylene is a plant hormone which can cause inhibition of seedling growth at low concentrations (Abeles, 1973) and is produced by many species of fungi in culture (Ilag and Curtis, 1968; Hutchinson, 1973). It is readily oxidised by potassium permanganate($KMnO_4$), boils at -103°C and freezes at -181°C.

After four days incubation, vapour from three litter containers and one control was assayed after passage through either the $KMnO_4$ trap or the -195°C trap. Four jars of 15 clover seedlings were treated per container for each trap. After a further seven days litter incubation the vapour was again assayed after passage through the -100°C trap.

Untreated vapour from litter incubated for four and eleven days caused inhibition (Table 10) of seedling growth. This inhibition was unaffected by the -100°C trap but was removed by the $KMnO_4$ and -195°C traps.

Ethylene has been shown to stimulate the growth of rice seedlings (Ku et al., 1970) so after four days incubation of a fresh collection of litter, vapour from three litter containers

Table 10 The inhibition of clover hypocotyl growth by litter vapour and the effect of $KMnO_4$ and cold traps on this response (B-T means in mm. Superscripts indicate differences at the 5% level.).

Time (days) of litter incubation	Container contents	Treatment of vapour			
		Untreated	$KMnO_4$	-195°C	-100°C
4	Control	19.52^{bc}	20.34^{abc}	23.15^{a}	
	Litter	5.03^{e}	21.17^{ab}	20.50^{ab}	
11	Control	19.92^{bc}			18.20^{c}
	Litter	5.23^{de}			5.46^{d}

C.V. = 12%

and one control was assayed using rice and clover seedlings. Six jars of ten seedlings were treated per container for each species. Clover growth was inhibited and rice growth stimulated (Table 11). Samples of five seedlings from each of the treatments were photographed (Fig. 3). Shortened, thickened hypocotyls of clover and the increased shoot growth and altered root form of rice can be seen.

Table 11 The effect of litter vapour on clover and rice seedling growth and the effect of the $KMnO_4$ trap on the response of rice seedlings (B-T means in mm. Superscripts indicate differences at the 5% level.).

Time (days) of litter incubation	Container contents	Untreated vapour		$KMnO_4$ treated vapour
		Clover	Rice	Rice
4	Control	19.00^{c}	19.15^{c}	
	Litter	4.56^{d}	25.65^{b}	
11	Control		19.90^{c}	20.34^{c}
	Litter		31.28^{a}	20.46^{c}

C.V. = 9%

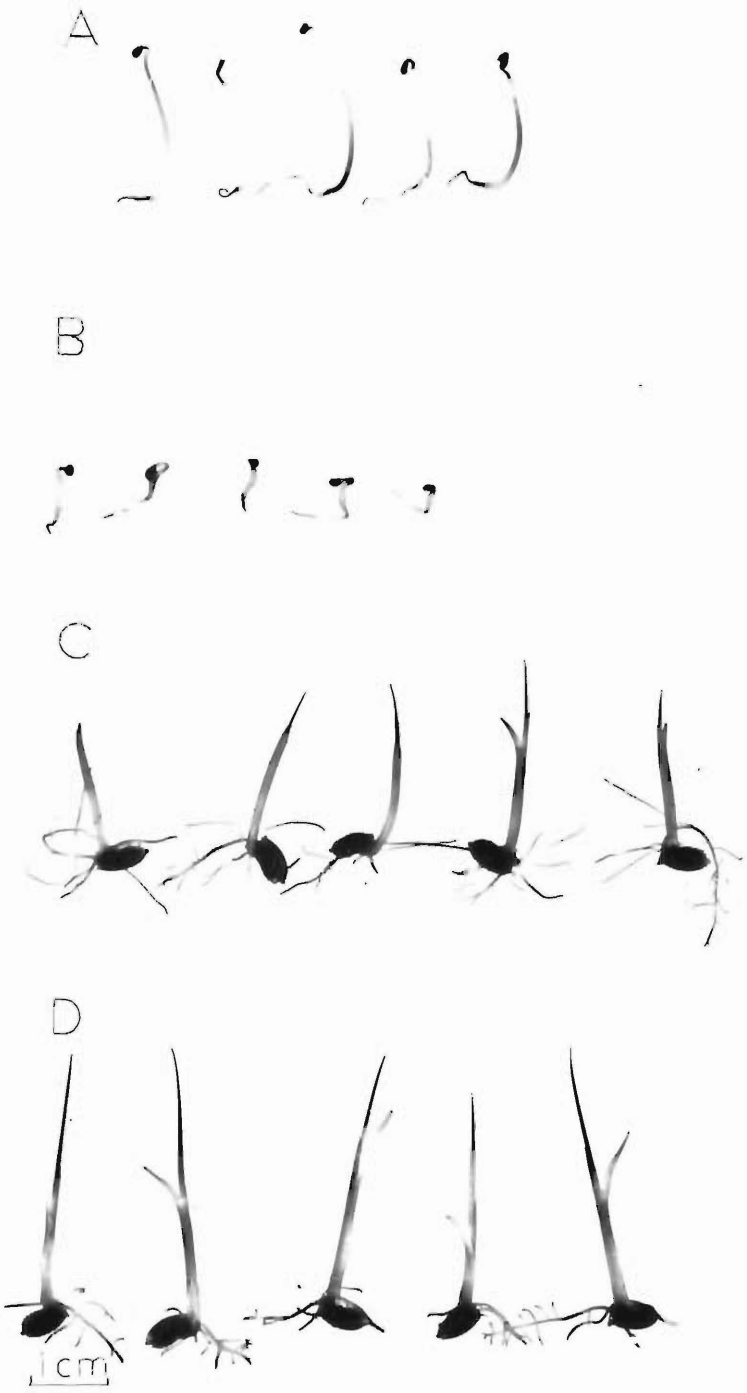


Fig. 3 Photograph of clover and rice seedlings treated with litter vapour.

A - Clover control

B - Clover treated with litter vapour

C - Rice control

D - Rice treated with litter vapour

After a further seven days incubation of the litter the vapour was again assayed using rice seedlings to check the effect of the $KMnO_4$ trap on the response. Rice growth was stimulated by untreated litter vapour and this stimulation was removed by the $KMnO_4$ trap (Table 11). These results are consistent with the reported effects of C_2H_4.

(6) The effect of C_2H_4 and C_2H_2 on clover seedling growth

Samples of litter vapour analysed on the gas chromatograph using Column A showed a peak with the same retention time as the C_2H_4 standard (Fig. 4a and b). But on this column there was very little difference in retention time between C_2H_4 and acetylene (C_2H_2) and as C_2H_2 can act as an analogue of C_2H_4 (Abeles, 1973) the following experiment was carried out.

A dilution of C_2H_2 to approximately 10ppm was prepared in an empty litter container. A similar dilution of C_2H_4 was prepared and the effect of these two gases on clover growth was compared with the effect of vapour from one litter container. Acetylene did not affect clover growth (Table 12), while C_2H_4 at the concentration used caused more inhibition than litter vapour.

Table 12 The effects of litter vapour, C_2H_2 and C_2H_4 on clover hypocotyl growth (B-T means in mm. Superscripts indicate differences at the 5% level.).

Control	Litter vapour	C_2H_2 (10.0ppm)	C_2H_4 (9.9ppm)
21.14^a	7.76^b	20.65^a	4.75^c

C.V. = 9%

Although C_2H_2 is not likely to be causing inhibition, if any is present in the litter vapour it could interfere with G.C.

Fig. 4 Gas chromatograph traces of litter vapour.

a. Column A, C_2H_2 (5ppm) and C_2H_4 (5ppm) x8

b. Column A, litter vapour x8

c. Column B, C_2H_6 (5ppm), C_2H_4 (5ppm) and C_3H_8 (5ppm) x8

d. Column B, litter vapour x8

e. Column C, C_2H_6 (1ppm), C_2H_4 (1ppm) and C_3H_8 (1ppm) x4

f. Column C, litter vapour x32

Peak representation: 1 - injection surge

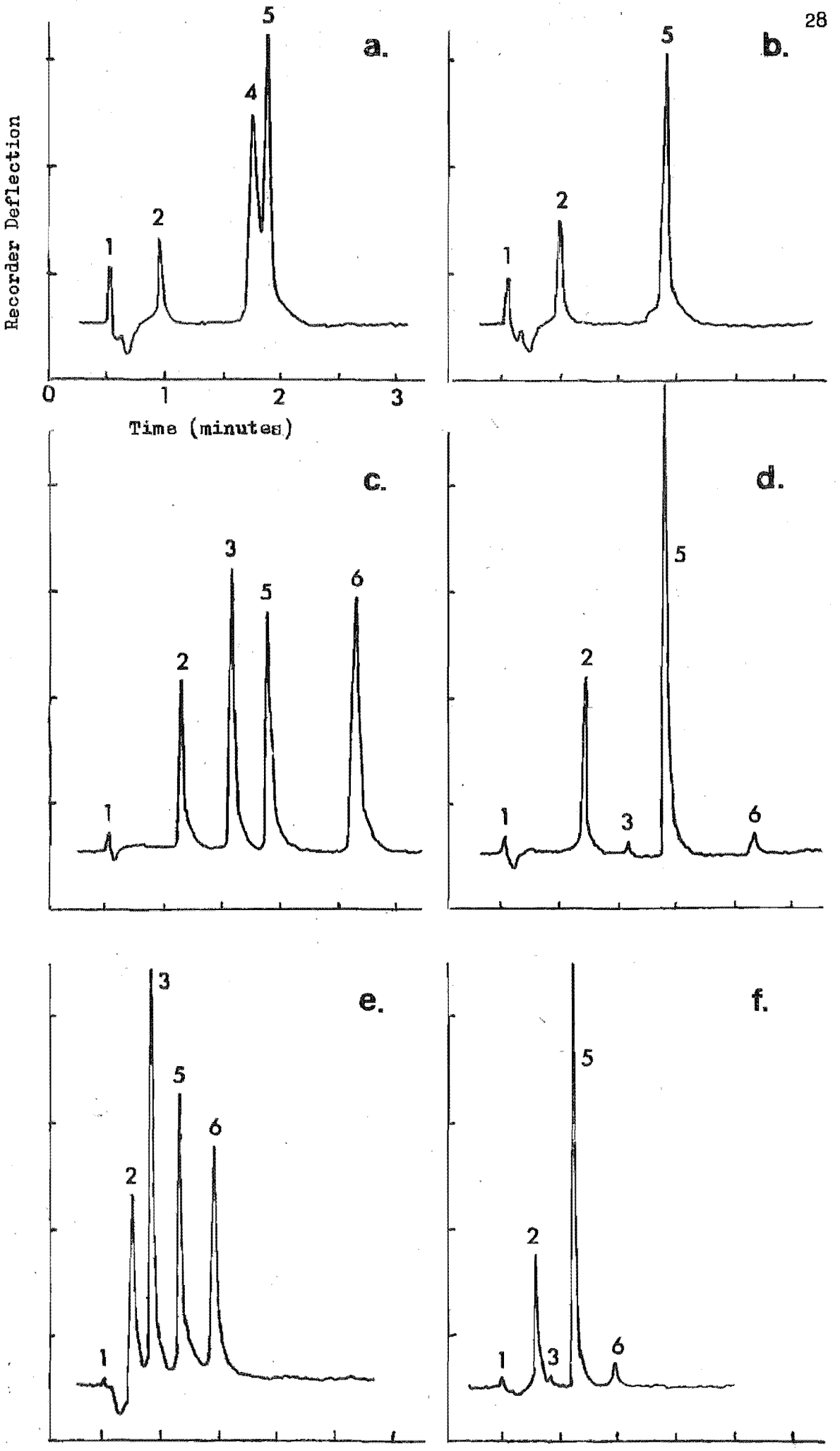
2 - air

3 - ethane (C_2H_6)

4 - acetylene (C_2H_2)

5 - ethylene (C_2H_4)

6 - propane (C_3H_8)


a.
b.
c.
d.
e.
f.
Recorder Deflection
Time (minutes)
0
1
2
3

analyses for C_2H_4 using Column A. To avoid this Column B was used in subsequent experiments.

(7) The effect of removal and subsequent readdition of C_2H_4 on growth inhibition of clover caused by litter vapour

After passage through a $KMnO_4$ trap, vapour from two litter containers and one control was assayed using clover seedlings. Vapour from a second group of containers was treated in the same way before assay, but C_2H_4 was injected into the assay jars before they were sealed. Five jars of 15 seedlings were used per container for each treatment. Vapour from one jar of each treatment was analysed on the G.C. using Column B. These jars were sealed with a rubber bung to enable the removal of the vapour sample.

Untreated litter vapour caused growth inhibition (Table 13). The $KMnO_4$ trap greatly reduced this inhibition and addition of C_2H_4 to $KMnO_4$ treated vapour completely restored the inhibitory effect. The concentration of C_2H_4 in the treated litter vapour ($KMnO_4$ trap + C_2H_4) was similar to the concentration in untreated vapour. $KMnO_4$ treated litter vapour had no detectable C_2H_4 (less than 0.03ppm).

Table 13 The effect of $KMnO_4$ treated litter vapour on clover growth and the change in response due to the subsequent addition of C_2H_4 to this vapour (B-T means in mm. Superscripts indicate differences at the 5% level.).

	Treatment of vapour					
Container contents	Untreated		$KMnO_4$ trap		$KMnO_4$ trap + C_2H_4	
	Growth	C_2H_4(ppm)	Growth	C_2H_4(ppm)	Growth	C_2H_4(ppm)
Control	19.91^b		23.37^a	0*	6.26^d	3.75
Litter	6.63^d	4.66	17.92^c	0*	6.71^d	3.30

* Not detected at 0.03ppm. C.V. = 17%

This experiment provides stronger evidence to support the theory that C_2H_4 produced by incubated P. radiata litter is causing the growth inhibition shown by clover seedlings.

*6

II. THE EFFECTS OF LITTER VAPOUR ON SEED GERMINATION

Seed germination is an essential step in the establishment of plants, so the effect of litter vapour on germination of clover, ryegrass and pine seed was investigated using the technique developed for seedling growth assays.

(1) The effect of litter vapour on the germination of clover and ryegrass

Vapour from four litter containers and four controls was assayed to establish its effect on clover germination. Each container was used to treat nine jars of 100 seeds after four days litter incubation and another nine jars after eleven days litter incubation. Germination was assessed in three jars at six periods after treatment with litter vapour. The vapour caused a delay in germination but by 48 h there was little difference between the control and litter vapour treatments (Fig. 5).

A similar experiment was carried out with ryegrass germination. Twelve jars of 100 seeds were treated for each container using the manifolds (described on page 10) in an attempt to reduce the variability caused by dilution of the vapour during treatment (discussed later). Germination was assessed at four intervals after vapour treatment and in all cases litter vapour caused a significant reduction in germination (Fig. 6). The difference was less after 96 h and it is probable that over a longer period that the difference would be reduced further. Ryegrass germination appears to be more sensitive to the litter vapour than clover

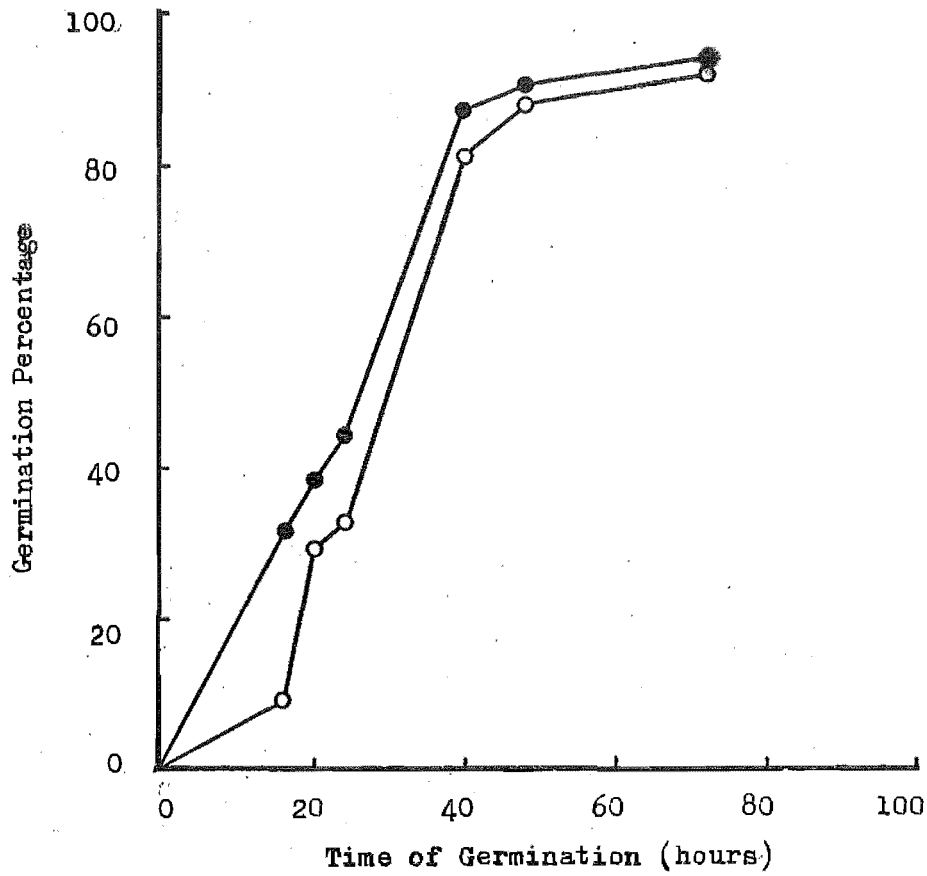


Fig. 5 Germination of clover in response to vapour from *P. radiata* litter.

● – Control

○ – Litter vapour

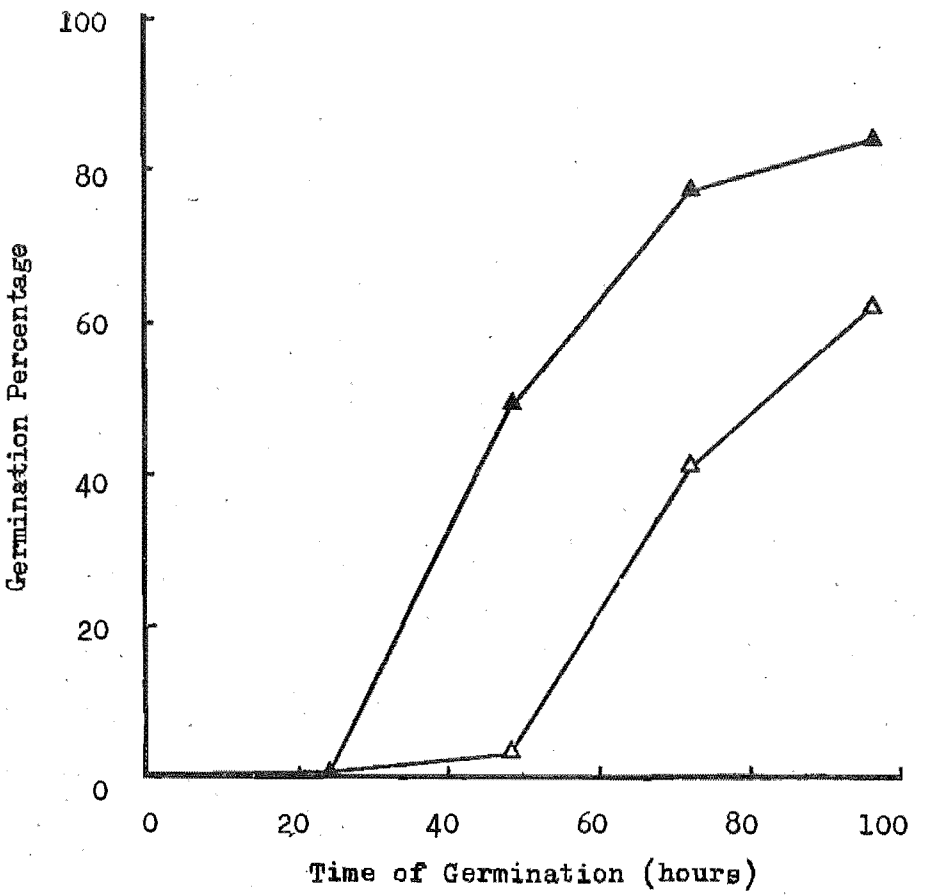


Fig. 6 Germination of ryegrass in response to vapour from *P. radiata* litter.

▲ – Control

△ – Litter vapour

germination.

(2) Germination of ryegrass and clover in response to diluted litter vapour

This experiment was carried out to obtain a dose-response relationship between litter vapour and germination. Vapour from four litter containers and four controls was assayed with clover seed after four days litter incubation and with ryegrass after a further seven days litter incubation. Dilutions by a factor of 10, 10^2 and 10^3 were set up in groups of three jars, each containing 100 seeds. A fourth group of three jars was treated with vapour in the usual way (without manifolds). Clover germination was assessed after 18 h and ryegrass germination after 48 h. Ryegrass germination was inhibited by both the undiluted vapour and the 10 fold dilution (Fig. 7). Clover germination was inhibited by undiluted vapour but stimulated by the 10 fold dilution and, at higher dilutions, litter vapour had no effect on the germination of either species.

(3) The effect of $KMnO_4$ treated litter vapour on the germination of clover, ryegrass and pine seeds

The trapping action of $KMnO_4$ was tested using vapour from four litter containers and four controls after four days incubation. Using the manifolds, six jars of 100 clover seeds were treated with vapour from the trap. Germination was assessed after 12 h and 18 h. The litter vapour was assayed again after a further seven days litter incubation and clover germination assessed after 18 h. Untreated litter vapour caused a marked stimulation of germination (Table 14). The $KMnO_4$ trap caused a slight reduction of the stimulatory effect (F test significant at 5% level) although this was not shown in the comparison of pairs of means using the S.N.K.

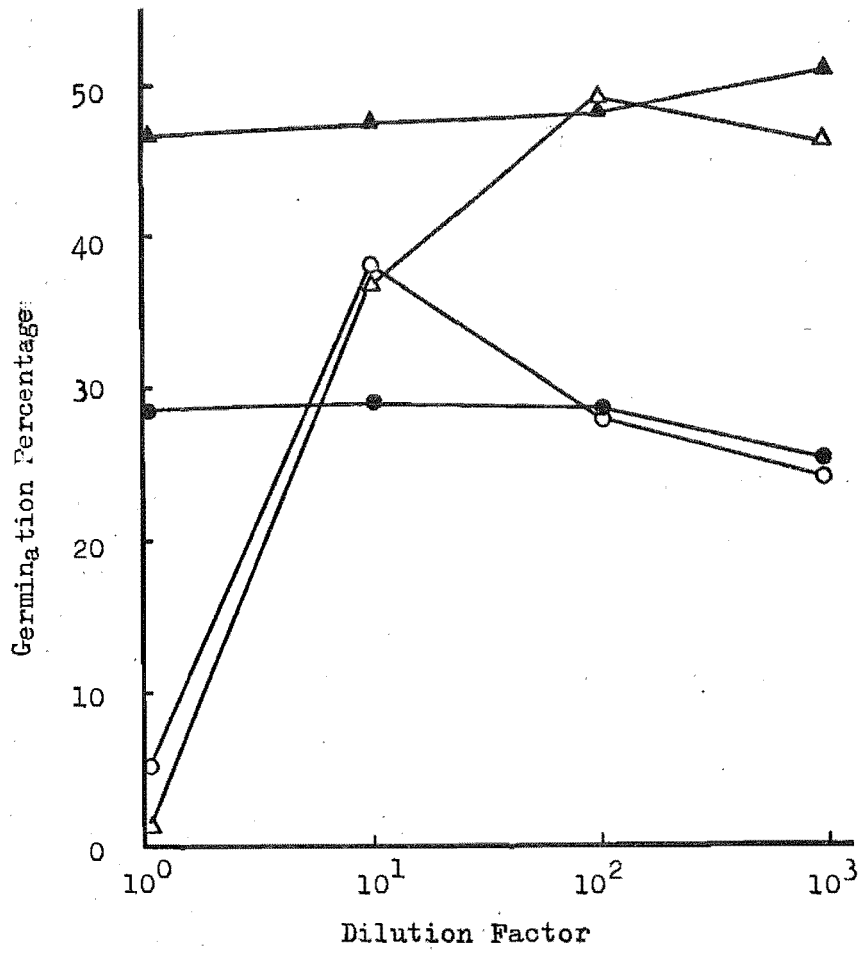


Fig. 7 Germination of clover and ryegrass in response to diluted vapour from P. radiata litter. Germination percentage was recorded after 18 h for clover and 48 h for ryegrass.

Clover ● – Control
○ – Litter vapour
Ryegrass ▲ – Control
△ – Litter vapour

test. The marked stimulation of clover germination directly contradicts the marked inhibition shown by undiluted litter vapour in the previous experiment (Fig. 7). The main difference between these experiments, apart from the use of different litter samples, was the use of the manifolds. Manifolds were not used in the previous experiment because only three jars of undiluted vapour were required for each container. This could have resulted in a difference in concentration of the litter vapour between the two experiments and hence a change in the response.

Table 14 The effect of $KMnO_4$ treated litter vapour on the germination of clover (B-T means in %. Superscripts indicate differences at the 5% level.).

Time (days) of litter incubation	Germination time (hours)	Container contents	Vapour treatment	
			Untreated	$KMnO_4$ trap
4	12	Control	3.3^{f}	3.8^{f}
		Litter	9.6^{e}	7.3^{e}
4	18	Control	20.8^{d}	20.9^{d}
		Litter	48.0^{a}	42.9^{ab}
11	18	Control	28.3^{c}	25.5^{cd}
		Litter	43.7^{ab}	37.9^{b}

C.V. = 17%

This experiment was repeated using fresh absorbent in the $KMnO_4$ trap. After incubation for four days, vapour from four containers of a fresh sample of litter and four controls was assayed after passage through the $KMnO_4$ trap. Six jars of 100 clover seeds were used per container for each treatment, and germination was assessed after 18 h. Untreated litter vapour stimulated germination (Table 15). The $KMnO_4$ trap removed this

stimulation and also reduced the germination in the control. It is probable that the trapping efficiency was increased by the use of fresh absorbent. Nevertheless the stimulatory response detected here was slight compared with the preceding experiment and these widely varying responses indicate some variable factor within the litter vapour or in the experimental conditions. *7

Table 15 The effect of $KMnO_4$ on the germination response of clover to litter vapour (B-T means in %. Superscripts indicate differences at the 5% level.).

Container contents	Vapour treatment	
	Untreated	$KMnO_4$ trap
Control	22.3^{b}	17.1^{c}
Litter	28.6^{a}	19.6^{bc}

C.V. = 16%

In a third experiment using the $KMnO_4$ trap the effect of litter vapour on ryegrass and pine germination was tested. After four days litter incubation vapour from four litter containers and four controls was assayed with three jars each of ryegrass and pine (50 seeds in each jar) at each manifold. Vapour to one manifold was passed through the $KMnO_4$ trap. Germination was assessed after two and nine days for ryegrass and pine respectively. Litter vapour inhibited germination of ryegrass and pine, but the $KMnO_4$ trap had no effect on this inhibition (Table 16). The response of ryegrass was similar to that detected previously (Figs. 6 and 7). *8

(4) The effect on the germination response of removing CO_2 from litter vapour

Accumulation of CO_2 in the litter containers during incub-

Table 16 The effect of $KMnO_4$ treated litter vapour on the germination of ryegrass and pine (B-T means in %. Superscripts indicate differences at the 5% level.).

Species	Container contents	Vapour treatment	
		Untreated	$KMnO_4$ trap
Ryegrass	Control	43.7^{a}	45.1^{a}
	Litter	8.0^{c}	5.7^{c}
Pine	Control	21.9^{b}	21.1^{b}
	Litter	6.3^{c}	5.1^{c}

C.V. = 19%

ation might have affected seed germination. Vapour from two litter containers and two controls was passed through a KOH trap and assayed with three jars each of clover and ryegrass (50 seeds in each jar). Germination was assessed after 18 h and 48 h for clover and ryegrass respectively. The inhibitory effect of the litter vapour on clover germination was completely removed by the KOH trap and, in the case of ryegrass, it was greatly reduced (Table 17).

Table 17 The effect on clover and ryegrass germination of litter vapour after removal of CO_2 (B-T means in %. Superscripts indicate differences at the 5% level.).

Species	Container contents	Vapour treatment	
		Untreated	KOH trap
Clover	Control	43.0^{bc}	41.2^{bc}
	Litter	27.7^{d}	39.9^{c}
Ryegrass	Control	53.2^{ab}	55.0^{a}
	Litter	19.3^{d}	41.8^{bc}

C.V. = 10%

An experiment was carried out to check the effect of CO_2

on germination. Three concentrations of CO_2 (0%, 1% and 10%) were applied to six jars each of clover and ryegrass (50 seeds in each jar). Germination of both species was inhibited by 10% CO_2, but there was no difference between the 0% and 1% treatments (Table 18). Ryegrass appeared to be more sensitive than clover.

Table 18 The effect of CO_2 on clover and ryegrass germination (B-T means in %. Superscripts indicate differences at the 5% level.).

% CO_2	Clover	Ryegrass
0	54.0^a	57.2^a
1	51.6^a	48.0^a
10	37.2^b	30.1^b

C.V. = 9%

(5) The response of germination to C_2H_4 and CO_2

To check the effect of C_2H_4 in combination with CO_2 on clover and ryegrass germination, a factorial experiment was set up with two concentrations of C_2H_4 and CO_2. Six jars of 50 seeds were used for each treatment combination and germination was assessed after 18 h and 48 h for clover and ryegrass respectively. The pattern of responses can be seen in the table of means and analysis of variance (Table 19). The main effect of CO_2 was a highly significant reduction in germination, but C_2H_4 showed no significant main effect. The significant interaction between species and C_2H_4 indicates a difference between clover and ryegrass in their response to C_2H_4 and the significant three way interaction shows that the change in response to C_2H_4 at different concentrations of CO_2 varies between species. In the absence of CO_2, C_2H_4 stimulates clover germination but inhibits ryegrass germination.

The presence of 10% CO_2 reduces germination and almost completely masks the effect of C_2H_4 in both species. Thus these results offer an explanation for the effects of litter vapour on germination. Clover germination was in some experiments stimulated but in others inhibited and ryegrass germination was always inhibited. A combination of CO_2 and C_2H_4 in the litter vapour could have caused these responses.

Table 19 The response of clover and ryegrass germination to CO_2 and C_2H_4.

(a) Seed germination (B-T means in %.).

% CO_2	C_2H_4 (ppm)	Clover	Ryegrass
0	0	40.9	52.5
	5	61.4	31.8
10	0	31.2	27.4
	5	36.3	25.7

(b) Analysis of variance.

Source of variation	Degrees of freedom	Mean square	F ratio	Significance level*
CO_2	1	1147.8	42.0	***
C_2H_4	1	2.2	0.1	N.S.
Species	1	285.9	10.5	**
CO_2 x C_2H_4	1	3.6	0.1	N.S.
CO_2 x Spp.	1	2.3	0.1	N.S.
C_2H_4 x Spp.	1	592.4	21.7	***
CO_2 x C_2H_4 x Spp.	1	291.8	10.7	**
Error	40	27.3		

* N.S. = not significant, ** = 1% level, *** = 0.1% level.

A further experiment was carried out to provide more information on the response of clover germination to mixtures of C_2H_4 and CO_2. Six concentrations of C_2H_4 were tested at four concentrations of CO_2 with one jar of 50 clover seeds per treatment. The experiment was replicated four times and germination assessed after 18 h. Four jars of seeds with no applied C_2H_4 and no KOH traps were used to check the effect of CO_2 produced by the seeds during germination. All jars were flushed with charcoal filtered air prior to treatment to remove traces of C_2H_4 which might be present in the jars. Ethylene concentrations are drawn on a logarithmic scale (Fig. 8) and the zero ppm treatment is marked as 0.01 ppm for convenience of graphing.

Ethylene had its maximum effect in the absence of CO_2 forming a plateau at 1.5 ppm. Carbon dioxide (1%) showed a stimulatory effect and this tailed off at 0.5 ppm C_2H_4 but the stimulation was less at higher concentrations of CO_2. This presents a complicated pattern of responses to C_2H_4 and CO_2, with germination being sensitive to low concentrations of both gases.

Effective concentrations of these gases are likely to be present both in controls and in litter vapour after trapping (assuming less than 100% efficiency) in $KMnO_4$ so the effects on clover germination recorded in previous experiments probably fall within this framework of response. * 9

III. ETHYLENE PRODUCTION BY P. RADIATA LITTER

These experiments were carried out to establish some of the conditions influencing C_2H_4 production by litter. A survey was made of C_2H_4 concentrations within the litter layer of two Canterbury Pinus radiata forests.

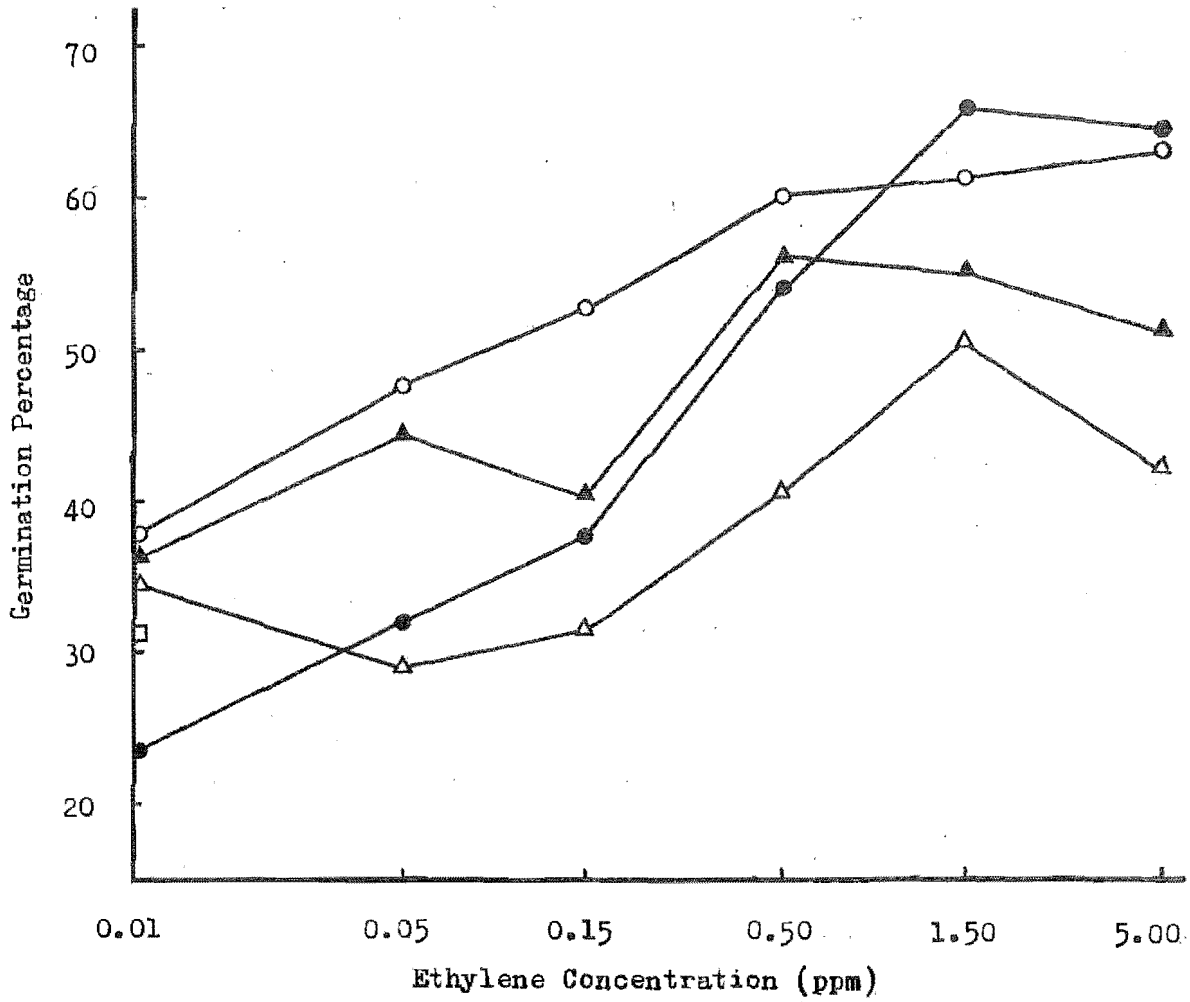


Fig. 8 The effect of C_2H_4 on the germination of clover at different concentrations of CO_2.

● – 0% CO_2
○ – 1% CO_2
▲ – 5% CO_2
△ – 10% CO_2
□ – Controll with no KOH trap

(1) The effect of autoclaving, tyndallising and air drying on C_2H_4 production by litter

A litter sample (40 cores) was mixed and subsamples (130 g) were placed in each of 12 jars. Four jars were not treated, four were autoclaved and four had 90% of their contents autoclaved. Samples of vapour were taken by syringe through rubber bungs and analysed daily on the G.C. using Column B. Unautoclaved litter showed a rapid production of C_2H_4 following a lag period of one day (Fig. 9). Although there was no further increase on the last day of measurement there was insufficient information to conclude that production was falling off.

Autoclaved litter had a higher initial concentration but produced C_2H_4 at a very much slower rate and the inoculated, autoclaved litter produced C_2H_4 at an intermediate rate.

To check the effect of a milder sterilisation treatment, 12 jars of litter were heated to 80°C for 30 min on three consecut- *10
ive days. The jars were sealed and samples of vapour analysed on the G.C. using Column C after zero, three and five days incubation. Tyndallised litter showed a similar pattern of C_2H_4 production to autoclaved litter, but production from untreated litter was slower in this experiment (Table 20) and the lag period was at least three days.

Table 20 The effect of tyndallisation on C_2H_4 production by litter.

Incubation time (days)	Tyndallised	Untreated
0	0.059 ppm	0*
3	0.640	0.611
5	1.249	8.889

* Less than 0.005 ppm.

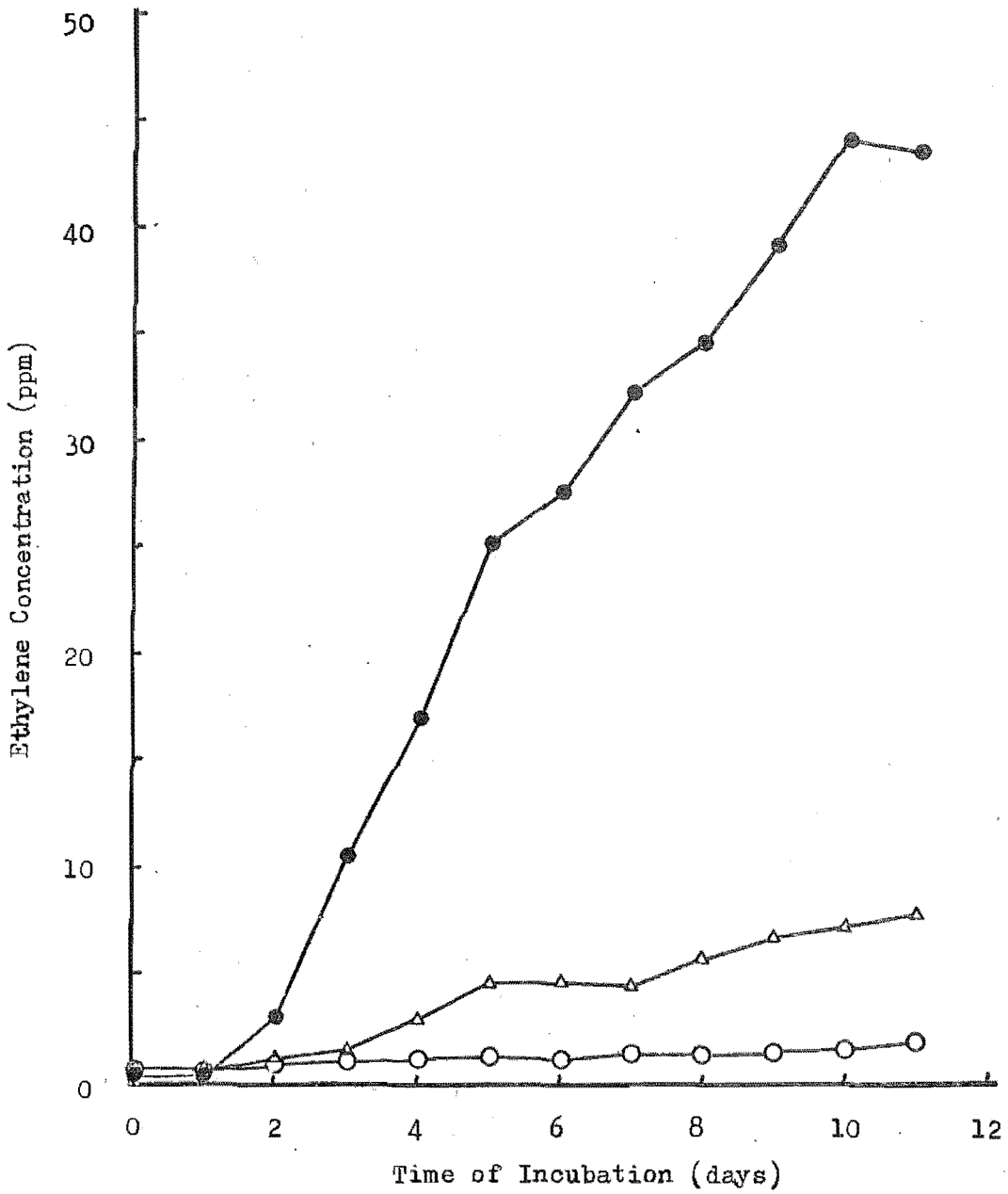


Fig. 9 The production of C_2H_4 by P. radiata litter and the effect of autoclaving on this process.

● – Untreated litter

△ – 90% autoclaved litter : 10% untreated litter

○ – Autoclaved litter

The effect of airdrying on C_2H_4 production was assessed by daily analyses (Column C) of vapour from six jars of air-dried litter. Ethylene production from air dried litter was similar to that of autoclaved and tyndallised litter (Fig. 10). The CO_2 content of the jars was measured after five days using titrimetric methods and was 0.62% and 7.75% for air dried and wet litter (moisture content 53% of wet wt.) respectively. This indicates that air-drying has drastically reduced respiratory activity.

(2) Disturbance effects under laboratory conditions

It is possible that C_2H_4 is produced merely as a response to the disturbance which occurs when the litter is sampled as higher plants frequently produce C_2H_4 in response to wounding or stress (Abeles, 1973). Six pairs of litter cores were cut and one of each pair was transferred to a jar with the least possible disturbance while the other core was broken up before transference. The jars were sealed and daily analyses made on the G.C. using Column C. The rubber bungs were then removed and the jars of litter were incubated for a 14 day 'rest' period after which the bungs were replaced and five daily analyses made. After a second 14 day 'rest' period, a disturbance treatment was applied to the litter which had not been disturbed previously and eight daily analyses were made. Because of the sampling procedure the variation was extremely high. Consequently the only significant differences between the disturbed and undisturbed litter occurred on the seventh and eighth day of the third set of analyses. But where the disturbance treatment had been applied (first and third set of analyses) it appeared to increase the rate of C_2H_4 production (Fig. 11). The lag period increased with each successive closure of the jars, possibly because of a slight drying of the

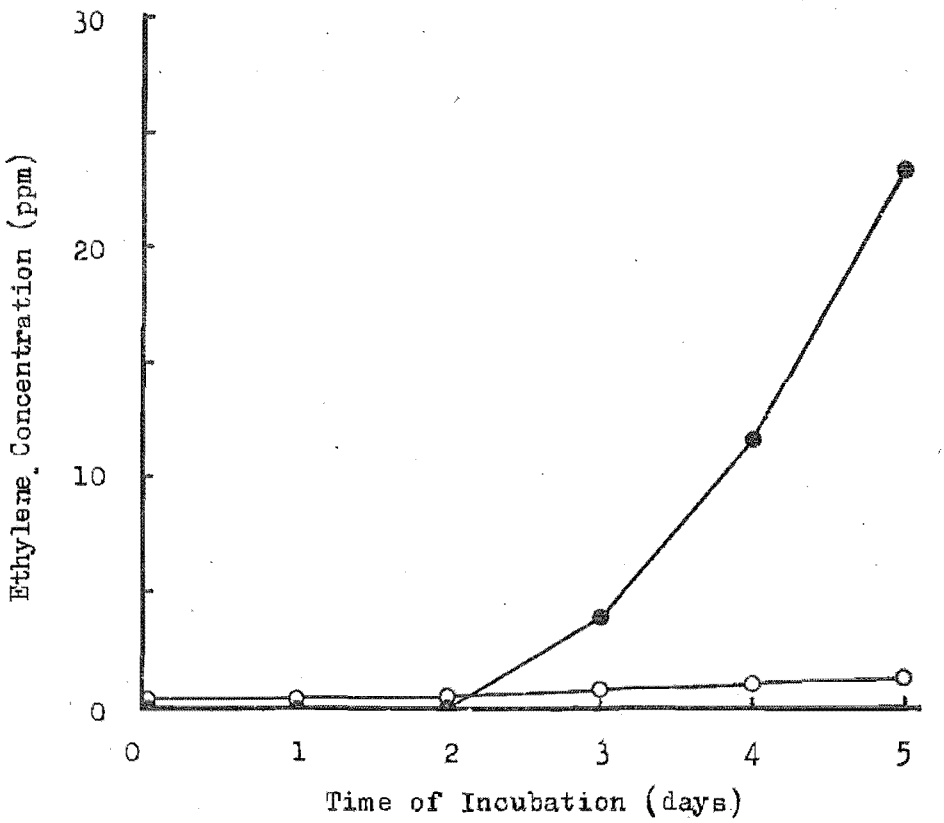


Fig. 10 Production of C_2H_4 by air dried P. radiata litter.

● - Untreated litter

○ - Air dried litter

Fig. 11 The effect of disturbance on the production of C_2H_4 by P. radiata litter.

A disturbance treatment was applied to six jars of litter (Group A) immediately after litter sampling for comparison with six jars of undisturbed litter (Group B). The vapour was analysed on three consecutive days after which the bungs were removed and the litter incubated for a 14 day 'rest' period. The jars were resealed and five daily analyses made before a second 14 day 'rest' period. Then the litter in the jars of Group B was disturbed, the jars sealed and eight daily analyses made.

○ - Group A immediately after sampling; disturbed

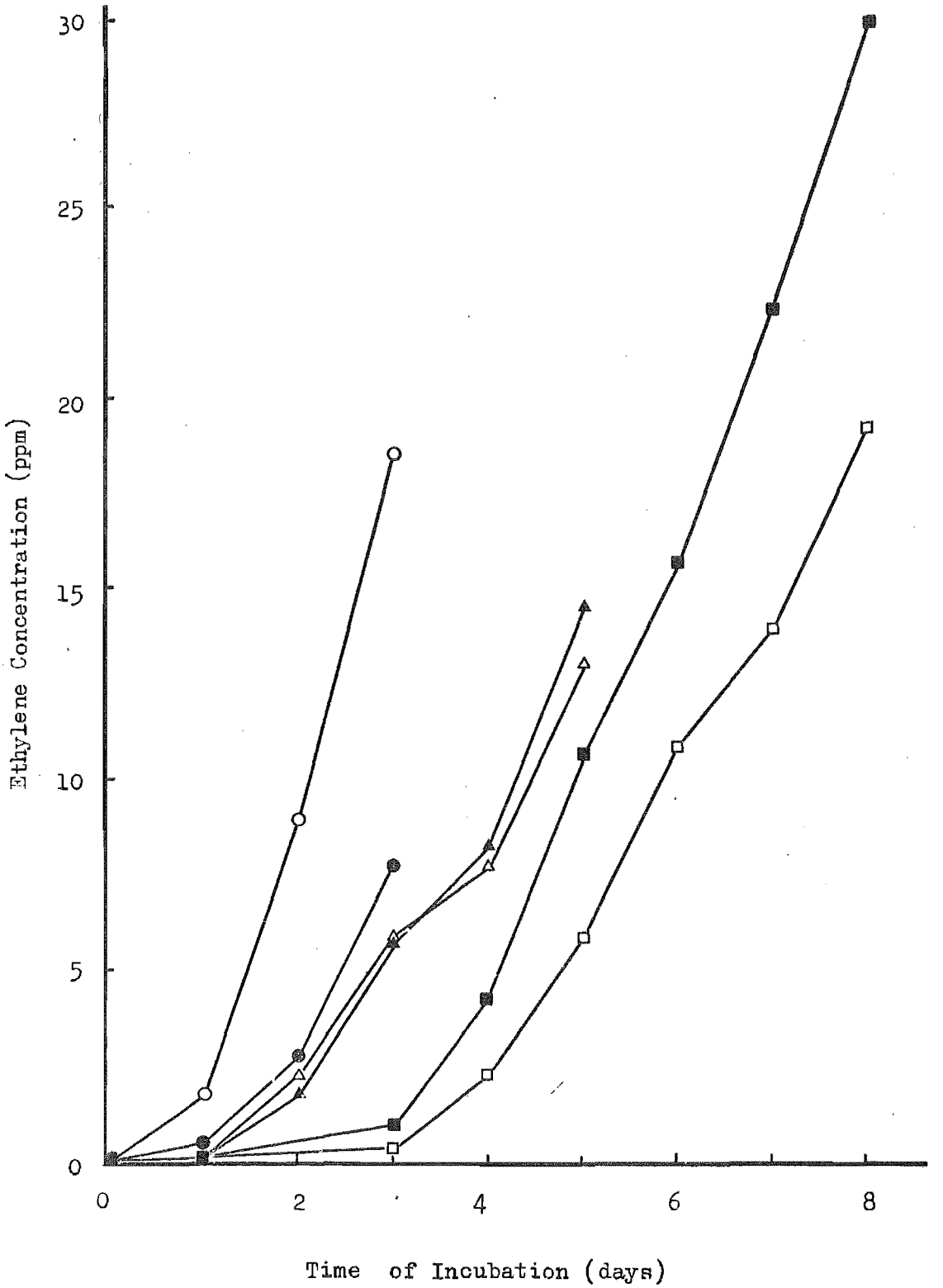
● - Group B immediately after sampling; undisturbed

△ - Group A after 14 day 'rest' period; undisturbed

▲ - Group B after 14 day 'rest' period; undisturbed

□ - Group A after second 14 day 'rest' period; undisturbed

■ - Group B after second 14 day 'rest' period; disturbed


30
25
20
15
10
5
0
Ethylene Concentration (ppm)
0
2
4
6
8
Time of Incubation (days)

litter during the 'rest' periods.

A second experiment with a disturbance treatment is described in the next section.

(3) The effect of removal of CO_2 and addition of O_2 on C_2H_4 production by litter

An attempt was made earlier to trap CO_2 within litter containers (page 23) but no conclusions could be drawn on the role of CO_2 in C_2H_4 production. An experiment was set up using the six jars of litter used previously for comparison with air dried litter. A vial containing 15 ml 0.8 N KOH was placed in each of three jars and O_2 was added to balance the pressure change caused by absorbtion of CO_2. In the presence of CO_2, C_2H_4 was produced rapidly (Fig. 12). There was only one day of 'rest' without bungs between experiments and this could account for the short lag period. In the jars in which CO_2 was trapped, C_2H_4 production was greatly reduced. Carbon dioxide concentrations measured on the fifth day of incubation were 1.56% and 6.47% respectively for jars with and without a KOH trap.

The 12 jars used as controls in the tyndallisation experiment were used in a second experiment testing the effect of CO_2 on C_2H_4 production. The jars were incubated without bungs for seven days to allow the litter to readjust to aerobic conditions * 13 and a factorial experiment was set up to test the effects of removal of CO_2 and of disturbance. Jars of litter in which CO_2 was allowed to accumulate produced C_2H_4 after a lag period of three days, but where CO_2 was trapped the concentration of C_2H_4 remained below the detection limit (0.005 ppm) until the eighth day of incubation (Fig. 13). By this time it is probable that the CO_2 traps were saturated. The disturbance treatment had no

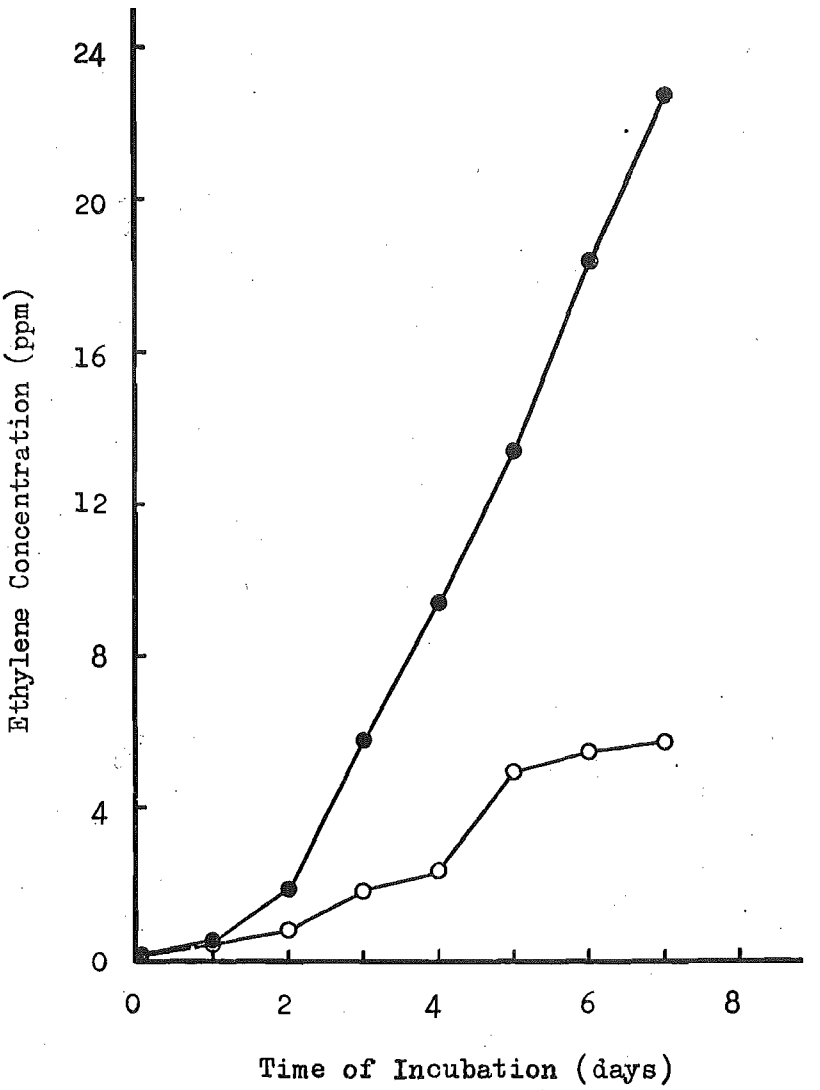


Fig. 12 Production of C_2H_4 by P. radiata litter as affected by removal of CO_2 and addition of O_2.

● - Untreated litter

○ - CO_2 removed, O_2 added

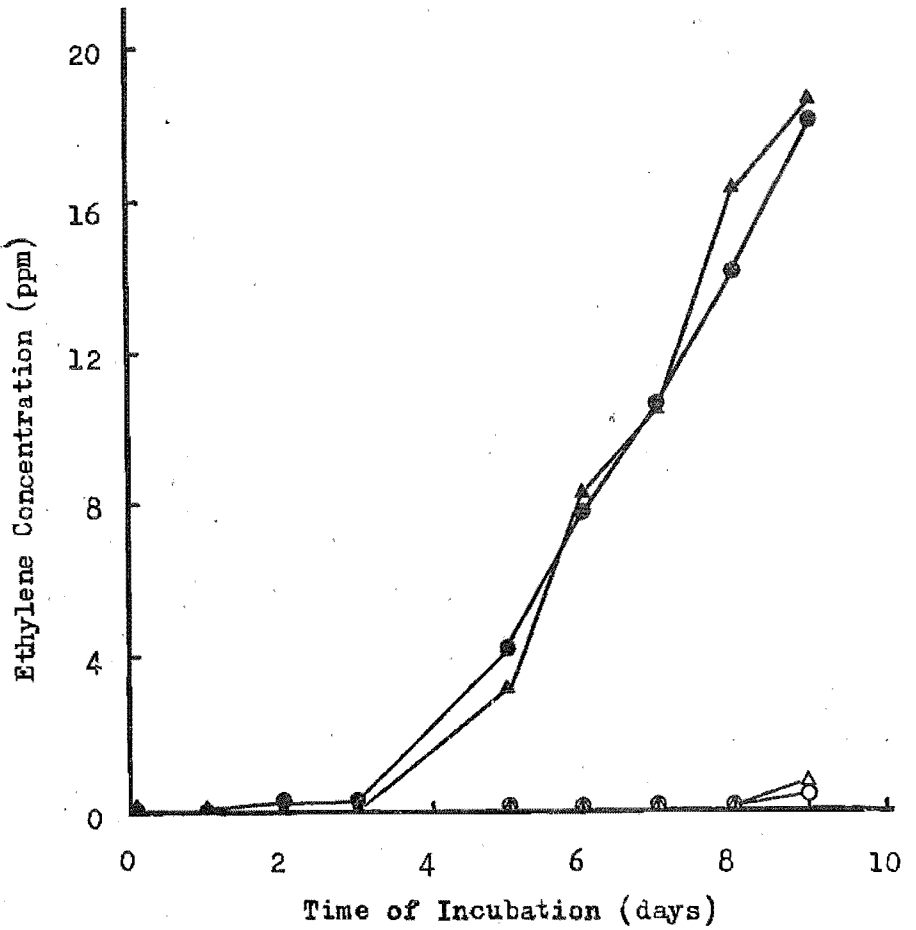


Fig. 13 The influence of disturbance on the production of C_2H_4 by P. radiata litter as affected by the removal of CO_2 and the addition of O_2.

● – Undisturbed; no gas treatment

△ – Undisturbed; CO_2 removed, O_2 added

▲ – Disturbed; no gas treatment

○ – Disturbed; CO_2 removed, O_2 added

detectable effect. The litter in this experiment had two weeks in which to recover from the disturbance caused by sampling and it is possible that this period was not sufficient. In the previous experiment on disturbance, the litter had five weeks to recover from sampling after which a slight effect from the disturbance treatment was detected. On the assumption that the litter will recover from disturbance in five weeks, it appears that litter can produce C_2H_4 in the absence of disturbance and that only a small increase in production results from disturbance.

(4) Field disturbance effects

Results from laboratory experiments on disturbance effects were not conclusive and so an experiment was set up in the field where sampling disturbance could be eliminated. Six pairs of one square metre plots were marked out in Ashley Forest. Three 2 ml gas samples were taken from near the bottom of the litter layer of each plot using plastic disposable syringes. On March 28th The litter from one plot of each pair was removed, broken up and replaced. Three gas samples were taken from each plot immediately after disturbance. Six samples of air at 1 m above the ground were taken as controls. Vapour from the plots was resampled after three days. The results (Table 21) indicate that no C_2H_4 accumulates in the litter layer in spite of a litter temperature of 18°C and moist conditions and that disturbance had no effect, although it is possible that the time interval allowed between samplings was unsuitable. Three days was the usual time taken from sampling to the end of the lag period under laboratory conditions. As it appears that disturbance does not play a major role in C_2H_4 production, no further work was carried out on this.

Table 21 The effect of disturbance on the C_2H_4 concentrations (ppm) in the litter layer of P. radiata at Ashley Forest.

Day	Control	Disturbed		Undisturbed
		Before	After	
0	0.010	0.006	0.006	0.007
3	0.013		0.012	0.013

(5) Ethylene in field samples from Ashley and Eyrewell Forests

Samples of vapour were taken from a range of sites at the two forests to assess the field concentrations of C_2H_4. Nine sites at Ashley and seven at Eyrewell were selected and six gas samples taken from near the bottom of the litter layer at each site. Three samples of air from 1 m above the ground were taken as controls for each site. The temperature of the litter at the time of sampling was 15°C and 10°C for Ashley and Eyrewell respectively. *-14 Details of climate and soil at each forest are given in Appendices 1 and 2.

The plastic and rubber of the syringes could affect the results by either absorbing or releasing C_2H_4 (Kavanagh and Postgate, 1970) so samples of 0.1 ppm C_2H_4 and charcoal filtered air were stored in plastic syringes for four hours before G.C. analysis using Column C. No effect was detected over this interval so the plastic syringes were used for field sampling.

No difference was detected between concentrations of C_2H_4 in the controls and in the litter vapour at any of the sites (Tables 22 and 23). At Eyrewell the controls were higher than at Ashley, possibly because of stubble fires in the vicinity of Eyrewell Forest at the time of sampling. The concentrations of C_2H_4 detected would unlikely to be having much affect on plant

growth (Abeles, 1973).

Table 22 Ethylene concentrations in field samples from Ashley Forest (detection limit 0.005 ppm)

Site	Mean C_2H_4 concentration (ppm)	
	Control	Litter layer
Clearfelled *P. radiata* (9 month)	0.006	0.007
Clearfelled *P. rad.* (2 y)	0.009	0.004
5 y regenerated *P. rad.*	0.012	0.008
11 y *P. radiata*	0.009	0.006
20 y *P. radiata*	0.008	0.006
28 y *P. radiata*	0.008	0.005
34 y *P. radiata*	0.009	0.008
31 y *P. nigra* var. *larica*	0.002	0.008
31 y *Pseudotsuga menziesii*	0.007	0.007

Table 23 Ethylene concentrations in field samples from Eyrewell Forest (detection limit 0.005 ppm)

Site	Mean C_2H_4 concentration (ppm)	
	Control	Litter layer
Clearfelled *P. radiata* (3 wks)	0.017	0.016
6 y *P. radiata*	0.017	0.016
12 y *P. radiata*	0.009	0.011
25 y *P. radiata*	0.011	0.018
41 y *P. radiata*	0.013	0.014
47 y *P. radiata*	0.013	0.009
13 y *P. nigra* var. *larica*	0.014	0.013

CHAPTER IV

DISCUSSION

Many compounds have been shown to be important in allelopathic relationships but most work has been concerned with water soluble substances and little consideration has been given to the role of volatiles in allelopathy. Muller W.H. and Muller C.H. (1964) have shown that volatile terpenes are involved in the allelopathy of Californian chaparral shrubs and several reports of plant growth inhibition by volatile compounds have been mentioned in Chapter I. The aim of this project was to establish if volatile components of Pinus radiata litter cause the reduced growth of second generation P. radiata at Nelson or affect pasture growth in integrated farm-forestry situations.

Few studies on this problem have been reported and no widely accepted technique has been established. One approach would be to use field trials but the problem of isolating the effects of volatiles from other factors and providing a realistic control presents difficulties. Trial plants would have to be kept separate from the soil and rain wash from the canopy and litter, yet be in sufficient contact with the litter to allow normal concentrations of volatiles to accumulate around the plants. The controls must also be isolated from the volatiles yet have the same conditions of humidity and air flow. Therefore assessment of the effects of volatiles was confined to the laboratory where more accurately controlled conditions could be provided. Enhanced accumulation of volatiles, which could be obtained by higher

temperatures and using closed containers with no soil as an absorptive sink, would facilitate their detection and identification. The concentrations of the identified inhibitors in samples of vapour from the field could then be measured and conclusions drawn on their likely influence on plant growth.

A laboratory technique described by Persidsky and Wilde (1954) and Muller W.H. (1965) tested a small quantity of material for volatile growth inhibitors by enclosing it in a container with bioassay seedlings. In the present study their technique was unsatisfactory because of inconsistency between experiments caused by the large variation in the litter layer in the field. The problem was overcome by using a litter sample made up of at least 20 smaller samples to provide vapour for assay. *15

One problem encountered with the technique was the dilution caused by the compressed air used to displace the vapour for assay. The assay jars were treated consecutively and the total volume of vapour displaced was approaching the volume of the gas space in the litter container. Gas mixing within the container would cause an inhibitor concentration gradient in the assay jars corresponding to the order in which they were treated. Seed *16 germination was sensitive to this gradient so manifolds were used to overcome the effect. Some dilution of the inhibitor would also occur as the vapour was flushed through the assay jars. One litre of vapour was passed into each jar to give a reasonable flushing action and some economy of vapour use. Because the vapour was displaced from the litter it was possible to pass it through absorbent and cold traps prior to assay and obtain useful information which, in combination with gas chromatographic analysis, led to the identification of the inhibitor.

The technique was applied to vapour from Pinus radiata litter

using the response of seed germination and seedling hypocotyl growth as indicators of inhibitory activity. These parameters were selected as being suitable for bioassay work and as essential processes in plant establishment. Effects on cell elongation and mitotic division in seedling hypocotyl growth may be related to similar processes ocurring in root and shoot extension of older plants.

Vapour from incubated Pinus radiata litter was shown to reduce the growth of clover, ryegrass and pine seedlings. The effect on clover was much more marked than on ryegrass and pine and was exhibited as shortened, thickened hypocotyls and radicles. On the clover radicles a short region dense with root hairs was formed suggesting a reduction in cell elongation. Burg and Burg (1965) found that the reduction in cell length caused by C_2H_4 was compensated for by an increase in radial growth, with the cell volume being the same in both controls and C_2H_4 treated seedlings. In clover seedlings treated with litter vapour the increased radial growth was not sufficient to account for the reduction in hypocotyl length. This implies a concurrent reduction in the rate of mitotic division. In ryegrass and pine the inhibition was less marked and it is possible that the reduced elongation is fully accounted for by an increase in radial growth.

Absorbent traps were used to aid identification of the inhibitory factor. The first absorbents used were water and paraffin wax and were aimed at removing water soluble metabolites and monoterpenes respectively from the litter vapour. Active microbial metabolism could give rise to inhibitory concentrations of products such as ethanol, acetaldehyde, acetone and butyric acid. Monoterpenes have been shown to be inhibitory to seed germination (Asplund, 1969) and they have been implicated in studies

on allelopathic relations between aromatic shrubs and herb species in Californian chaparral (Muller W.H. and Muller C.H., 1964). Pine resin is rich in monoterpenes and hence their occurrence in the litter atmosphere is possible. The traps did not reduce the inhibition, suggesting that compounds soluble in water and paraffin wax are not involved. For these results to be meaningful it is necessary to assume sufficient trapping efficiency to reduce the concentration of inhibitory substances to a level which has no effect. It was possible to demonstrate that the wax trap reduced the concentration of a representative monoterpene, camphor, from a level inhibitory to germination (Asplund, 1969) to below the detection threshhold of human smell. Both water and wax traps caused a slight increase in the inhibitory effect. The most likely reason for this is the sequence in which the treatments were applied. The assay jars were treated consecutively and, because it was impractical to treat every alternate jar with vapour which had passed through the trap, it was necessary to apply the treatment to the vapour in a particular order. It was decided to pass the first vapour displaced from the litter containers through the trap because any reduction in the inhibition by the trap could then be assigned with more certainty to absorbtion of an active component. If the trap did not absorb the inhibitor then any dilution of the vapour by the compressed air would be shown as a decrease in the inhibition caused by the untreated vapour. An alternative explanation is that some other component is being absorbed, resulting in an increase in the concentration of the inhibitor. It seems unlikely that this would occur.

Prior to assay of litter vapour, the litter was incubated for four days in a closed container. During this period, respiratory activity could raise the concentration of CO_2 to inhibitory

levels so experiments aimed at investigating this and the effect of the corresponding O_2 depletion were carried out. The results again indicate an increase in inhibition caused by the litter vapour after passage through the trap, which argues against CO_2 as a cause of growth inhibition. Possible reasons for this increase in inhibition have been discussed above. The growth inhibition was not caused by lowered O_2 concentrations in the litter vapour so the investigation was directed at other inhibitory volatiles.

Results of experiments using $KMnO_4$ and cold traps indicated that C_2H_4 could be the inhibitor. Ethylene is well known for its role in fruit ripening but it also has other effects on plant physiology, including the inhibition of seedling growth (Abeles, 1973). It is produced by many species of fungi, bacteria and actinomycetes (Ilag and Curtis, 1968; Lynch, 1972; DaSilva et al., 1974; Smith A.M. and Cook, 1974) and has been detected in soil at concentrations sufficient to affect plant growth (Smith K.A. and Restall, 1971; Smith A.M. and Cook, 1974). The cold traps are not specific for C_2H_4 and $KMnO_4$ will trap any compound which is readily oxidised so further evidence for the presence of C_2H_4 was needed. Mercuric perchlorate was used as an absorbent in a trap specific for olefins but it had no effect on inhibition. Using the gas chromatograph it was possible to check the efficiency of this absorbent for trapping C_2H_4 and it was found to reduce the concentration by only 20% under the experimental conditions used and the C_2H_4 remaining would account for the unchanged inhibition. *17

Rice seedlings were used to provide confirmatory evidence that C_2H_4 was involved in the growth inhibition. Ku et al. (1970) reported stimulation of the growth of rice coleoptiles by C_2H_4.

The stimulation of rice seedling growth caused by litter vapour was removed when the vapour was passed through the $KMnO_4$ trap. The measurements included the whole shoot and hence the response cannot be attributed to any particular part. Roots of rice seedlings treated with litter vapour appeared thicker and had a greater formation of laterals than control seedlings. Ethylene stimulates the formation of root initials (Abeles, 1973) and this could account for the increased development of laterals in the rice seedlings.

Further evidence for the presence of C_2H_4 in litter vapour was provided by gas chromatographic analysis and the effect of C_2H_4 alone indicated its potency. Removing C_2H_4 from litter vapour using the $KMnO_4$ trap and replacing it with commercial C_2H_4 fully restored the inhibitory activity. This suggests that C_2H_4 is responsible for all the inhibition of seedling growth caused by the litter vapour.

Effects of litter vapour on seed germination were also assessed. Seed germination is essential in plant establishment but it is a different physiological process to seedling growth and has different control mechanisms. Litter vapour caused a reduction in the germination of both ryegrass and pine seed. Clover seed germination showed both stimulatory and inhibitory responses in different experiments. In the case of clover, the germination reduction was a transient delay rather than a reduction in total germination. A similar situation probably occurs with ryegrass because, although the time periods in the experiment were not long enough to show this conclusively, the difference between the germination of the control and the litter vapour treated seed decreased with time.

The effect of dilutions of the litter vapour gave more

information on the apparently contradictory responses of clover germination. Full strength vapour caused a reduction in germination and a ten fold dilution caused a stimulation. Further dilution by a factor of ten reduced the effect below the detection limit. This type of response could be caused by a single compound which is inhibitory at high concentrations but which is stimulatory at lower concentrations. Alternatively it could be a response to a combination of two substances, one of which is inhibitory and, at high concentrations, masks a stimulatory effect of the second compound. On dilution the inhibitory effect might be so reduced that it allows stimulation by the second compound to be exhibited.

An attempt was made to identify the active components using the absorbent traps. The $KMnO_4$ trap slightly reduced the stimulatory effect of litter vapour on clover germination but this trap had no effect on the reduced germination of ryegrass and pine. This suggests that C_2H_4 may be involved in producing the stimulation of clover germination but that the trap is not lowering the concentration sufficiently to completely remove the effect. Ethylene, which inhibits seedling growth, is reported to have a role in breaking dormancy and hence stimulating germination (Stewart and Freebairn, 1969). These two effects appear contradictory in that a seed which germinates in response to C_2H_4 will also have its subsequent growth reduced, lessening its chance of survival.

The germination in controls was also reduced by passage of the control vapour through the $KMnO_4$ trap and it is possible that C_2H_4 present in the laboratory in which the experiment was set up (0.1 ppm) was sufficient to stimulate clover germination. Ethylene did not appear to be causing germination inhibition and it is probable that some other compound in the litter vapour is

active in this respect.

Although not affecting seedling growth, accumulated CO_2 could be influencing seed germination. Measurement of CO_2 concentrations within litter containers using titrimetric methods indicated at least 6% - 8% CO_2 in the litter vapour. Removal of CO_2 from litter vapour by a KOH trap markedly reduced the germination inhibition.

Both C_2H_4 and CO_2 alone were shown to stimulate clover germination but this response was markedly modified by the presence of the other gas. Egley and Dale (1970) found that the maximum stimulation of witchweed germination caused by 0.1 ppm C_2H_4 did not occur in the presence of 10% CO_2 and Ballard (1958) reports a dormancy breaking effect of CO_2 on subterranean clover.

In germination experiments with litter vapour no precautions were taken to remove CO_2 and C_2H_4 from the controls. As the concentrations of these gases probably vary in both the control and the litter vapour between different experiments, the effect of these two gases could explain the contradictory results obtained for clover germination.

Ryegrass germination was inhibited by both CO_2 and C_2H_4 and these compounds are probably causing at least part of the germination inhibition caused by litter vapour. This is not in agreement with the theory that C_2H_4 acts as a germination stimulator (Stewart and Freebairn, 1969; Abeles, 1973). The germination inhibition of ryegrass (in Table 16, Figs. 6 and 7) and clover (Fig. 7) in response to litter vapour appears to be greater than can be explained by a combination of CO_2 and C_2H_4 and it is possible that some other component of the vapour is acting synergistically with these gases. Carbon dioxide and C_2H_4 do appear to be involved in the effects observed and, if present in

the field in sufficient concentrations, could affect germination patterns in Pinus radiata forests.

Ethylene production in pine litter could arise from senescent leaf tissue, microbial activity or the non-enzymic breakdown of decomposition products. Since heat stops the production of C_2H_4, non-enzymic reactions are probably not involved unless C_2H_4 is formed by the spontaneous reaction of a metabolic intermediate. In this case heat would affect the system producing the intermediate and hence suppress the production of C_2H_4. Senescent leaves may continue to metabolise for some time after leaf fall and during this time may produce C_2H_4. But micro-organisms can also produce C_2H_4 and, as they are probably responsible for a majority of the metabolic activity of the litter, are the most likely source of C_2H_4 production. *18

Injury to higher plants can result in C_2H_4 production (Abeles, 1973) so it is possible that cutting litter samples may induce the C_2H_4 production shown by the litter. The results of experiments studying the effects of disturbing litter suggested that the disturbance might be increasing C_2H_4 production slightly, possibly by making availiable new pools of substrate.

Accumulation of CO_2, or the depletion of O_2, does appear to be involved in C_2H_4 production. The lag period during which C_2H_4 concentrations are small or not detectable followed by a rapid production suggests that conditions developing within the containers induce the C_2H_4 producing systems. Removal of CO_2 and addition of O_2 to the litter atmosphere completely suppressed C_2H_4 production. The increase in C_2H_4 concentration did not appear to follow a logarithmic relationship and it may be that an existing population is switched on to a constant rate of production at a threshhold concentration of CO_2 or O_2.

An interesting feature of C_2H_4 production by litter is the very low concentration of C_2H_4 during the lag period. Even though the experiments were set up in a laboratory in which the C_2H_4 concentration was about 0.1 ppm, the initial C_2H_4 concentration in vapour from moist litter was often below the detection limit (i.e. less than 0.005 ppm). Air dried litter had an initial concentration (0.27 ppm) somewhat higher than laboratory air, suggesting that some mechanism is actively removing C_2H_4 from the litter atmosphere. An aerobic biologically mediated system has been implicated in the absorption of C_2H_4 by soil (Abeles et al., 1971) and it is possible that a similar system is operating here.

Ethylene biosynthesis by higher plants and fungi is generally regarded as an aerobic process but synthesis by bacteria under anaerobic conditions has also been shown (Smith A.M. and Cook, 1974). In the absence of suitable facilities it was not possible to monitor CO_2 and O_2 and consequently no information can be given on the concentrations of these gases at the onset of C_2H_4 production. It is possible that both the C_2H_4 producing systems and the suggested active removal system are aerobic but have different requirements for O_2 or sensitivities to CO_2. In this situation C_2H_4 would be produced under normal conditions but the removal system would prevent accumulation. Deactivation of this by changing atmospheric conditions would allow accumulation of C_2H_4 if the changes did not affect the C_2H_4 producing systems as well.

It can be concluded that conditions developing within the litter containers induce the accumulation of C_2H_4, and that these conditions may not generally operate in the field.

Some means of accumulation are necessary for an inhibitory volatile compound to affect plants in the field. The litter layer, consisting of loosely packed needles and twigs, is, on

average, 6.4 cm deep. The resistance to diffusion of gases into the surrounding air would be small and, unless the rate of C_2H_4 production was high, little accumulation would be expected. Muller C.H. and del Moral (1966) suggested that terpenes from Californian chaparral shrubs accumulated by absorption onto soil colloids and were subsequently transferred to germinating seedlings by solution in their cuticular lipids but no such pathway can be envisaged here. Ethylene may be absorbed by the soil but Abeles et al. (1971) suggest that this is an irreversible process. In any case, C_2H_4 produced in the litter would be more likely to diffuse along the less restrictive path into the air than downward into the soil.

A survey of C_2H_4 in samples of litter vapour from the field showed concentrations similar to those in the air. Vapour samples were taken from near the bottom of the litter horizon using plastic disposable syringes. It is possible that some dilution from the atmosphere could occur as the sample is drawn into the syringe but the air space of the litter layer is large and therefore should provide a two millilitre sample with negligible dilution. It is possible that conditions prevailing may not have been suitable for high rates of C_2H_4 production. At Ashley Forest the litter temperature was 15°C and the litter was moist when the survey was carried out but at Eyrewell the litter temperature was lower (10°C). Vapour samples were also taken at Ashley when the litter temperature was 18°C but no C_2H_4 was detected though active microbial metabolism would be expected at this temperature under moist conditions.

If C_2H_4 is produced in the litter layer in Pinus radiata forests, it did not accumulate under the conditions prevailing at the time of the field surveys. This could be because diffusion

from the litter keeps pace with C_2H_4 production but it is more likely that the concentrations of CO_2 and O_2 necessary for C_2H_4 accumulation do not develop under field conditions. Reduced diffusion could occur in pockets of very deep litter and under wet conditions when C_2H_4 may accumulate. Intensive monitoring of C_2H_4 throughout the year is needed to provide enough information to make a definite statement on its occurrence in the field, but at this stage it appears that C_2H_4 does not normally accumulate in the litter layer of P. radiata forests in Canterbury.

In the laboratory, all the inhibition of seedling growth caused by litter vapour can be accounted for by the C_2H_4 present so it appears that other volatiles have little or no effect. This study has been restricted to the litter horizon but decomposition of roots within the mineral soil could generate sufficient inhibitory volatiles to affect plant growth. This hypothesis could account for at least part of the reduced growth of second generation P. radiata in Nelson, but volatiles from surface litter would be unlikely to affect tree growth. Similarly, volatiles from litter are not likely to be affecting the growth of pasture swards under stands of P. radiata.

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REFERENCES

ABELES, F.B. Ethylene in plant biology. New York and London, Acad. Press, 1973, 302 p.

ABELES, F.B., CRAKER, L.E., FORRENCE, L.E. and LEATHER, G.R. Fate of air pollutants: removal of ethylene, sulphur dioxide and nitrogen dioxide by soil. Science 173, 1971: 914-916.

ADAMS, J.A. The nutritional status of some soils of the Nelson area in relation to the growth of Pinus radiata. N.Z. Soil News 19, 1971: 157-167.

AL-NAIB, F.A. and RICE, E.L. Allelopathic effects of Platanus occidentalis. Bull. Torrey bot. Club 98, 1971; 75-82.

APPLETON, E.J. and SLOW, L.J. Nutritional disorders and fertiliser trials in Pinus radiata stands in Waimea county, Nelson. N.Z. Jl. For. 11, 1966: 185-201.

ASPLUND, R.O. Quantitative aspects of the phytotoxicity of monoterpenes. Weed Sci. 17, 1969: 454-455.

BALLARD, L.A.T. Studies of dormancy in the seeds of subterranean clover I. Breaking of dormancy by CO_2 and by activated carbon. Aust. J. biol. Sci. 11, 1958: 246-260.

BEDNALL, B.H. The problem of lower volumes associated with second rotations in Pinus radiata plantations in South Australia. Woods and Forests Dept., South Australia, Bull. No. 17, 1968.

BEVEGE, D.I. Inhibition of seedling hoop pine (Araucaria cunninghamii Ait.) on forest soils by phytotoxic substances from the root zones of Pinus, Araucaria and Flindersia. Pl. Soil 29, 1968: 263-273.

BODE, H.R. Allelopathy in some Juglandaceae. Planta 51, 1958: 440-480.

BONNER, J. The role of toxic substances in the interactions of higher plants. Bot. Rev. 16, 1950: 51-65.

BORNER, H. Liberation of organic substances from higher plants and their role in the soil sickness problem. Bot. Rev. 26, 1960: 393-424.

BROWN, R.T. Influence of naturally occurring compounds on the germination and growth of Jack Pine. Ecology 48, 1967: 542-546.

BURG, S.P. and BURG, E.A. Ethylene action and the ripening of fruits. Science, N.Y. 148, 1965: 1190-1196.

DAL NOGARE, S. and JUVET, R.S. Gas liquid chromatography. New York, Wiley, 1965, p. 253.

DASILVA, E.J., HENRIKSSON, E. and HENRIKSSON, L.E. Ethylene production by fungi. Pl. Sci. lettens 2, 1974: 63-66.

DE BELL, D.S. Phytotoxins - new problems in forestry? J. For. 68, 1970: 335-337.

DE BELL, D.S. Phytotoxic effects of cherry bark oak. Forest Sci. 17, 1971: 180-185.

DEL MORAL, R. and CATES, R.G. Allelopathic potential of the dominant vegetation of western Washington. Ecology 52, 1971: 1030-1037.

DEL MORAL, R. and MULLER, C.H. Fog drip: a mechanism of toxin transport from Eucalyptus globulus. Bull. Torrey bot. Club 96, 1969: 467-475.

EGLEY, G.H. and DALE, J.E. Ethylene, 2-chloroethyl phosphonic acid and witchweed germination. Weed Sci. 18, 1970: 586-589.

FLORENCE, R.G. Decline of old growth redwood forests in relation to some soil microbiological processes. Ecology 46, 1965: 52-64.

FLORENCE, R.G. Factors that may have a bearing upon decline of productivity under forest monoculture. Aust. For. 31, 1967: 50-71

FLORENCE, R.G. and CROCKER, R.L. Analysis of blackbutt (Eucalyptus pilularis Sm.) seedling growth in a blackbutt forest soil. Ecology 43, 1962: 670-679.

GLEN, A.T. and HUTCHINSON, S.A. Contribution of triacetylene to the biological effects of Fomes annosus. Trans. Br. mycol. Soc. 61, 1973: 583-585.

GRANO, C.X. Is litter a barrier to the initial establishment of shortleaf and loblolly pine reproduction? J. For. 47, 1949: 544-548.

HALLS, L.K. and SUMAN, R.F. Improved forage under southern pines. J. For. 52, 1954: 848-851.

HAMMERSCHLAG, F.A. and LINDERMAN, R.G. Effect of five acids from pine needles on the ecology of Fusarium oxysporum F. sp. lilii in non-sterile soil. Proc. Am. phytopath. Soc. 1, 1974: 150.

HOLM, R.E. Volatile metabolites controlling germination in buried weed seeds. Pl. Physiol., Lancaster 50, 1972: 293-297.

HUTCHINSON, S.A. Biological activities of volatile fungal metabolites. A. Rev. Phytopath. 11, 1973: 223-246.

HUTCHINSON, S.A. and COWAN, M.E. Identification and biological effects of volatile metabolites from cultures of Trichoderma harzianum. Trans. Br. mycol. Soc. 59, 1972: 71-77.

ILAG, L. and CURTIS, R.W. Production of ethylene by fungi. Science, N.Y. 159, 1968: 1357-1358.

JAMESON, D.A. Degradation and accumulation of inhibitory substances from Juniperus osteosperma. Pl. Soil 33, 1970: 213-224.

KAVANAGH, E.P. and POSTGATE, J.R. Absorbtion and release of hydrocarbons by rubber closures: a source of error in some biological assays. Lab. Pract. 19, 1970: 159-160.

KEEVES, A. Some evidence of loss of productivity with successive rotations of Pinus radiata in the south east of South Australia. Aust. For. 30, 1966: 51-63.

KNOWLES, R. and LAISHLEY, E. Factors in forest tree litter extracts affecting the growth of soil micro-organisms. Nature, Lond. 184, 1959: 1169.

KOROLEFF, A. Leaf litter as a killer. J. For. 52, 1954: 178-182.

KOWAL, N.E. Effect of leaching on pine litter decomposition rate. Ecology 50, 1969: 739-740.

KU, H.S., SUGE, H., RAPPAPORT, L. and PRATT, H.K. Stimulation of rice coleoptile growth by ethylene. Planta 90, 1970: 333-339.

LYNCH, J.M. Identification of substrates and isolation of micro-organisms responsible for ethylene production in soil. Nature, Lond. 240, 1972: 45-46.

MALISZEWSKA, W. and MOREAU, R. A study of the fungal microflora in the rhizosphere of fir (Abies alba Mill.). In Parkinson, D. and Waid, J.S. The ecology of soil fungi. Liverpool, Liverpool Univ. Press, 1960: 209-218.

MARSHALL, A.M. and HUTCHINSON, S.A. Biological activity of volatile metabolites from cultures of Fomes scutellatus. Trans. Br. mycol. Soc. 55, 1970: 239-251.

MEIGH, D.F., FILMER, A.A. and SELF, R. Growth inhibitory volatile aromatic compounds produced by Solanum tuberosum tubers. Phytochem. 12, 1973: 987-993.

MULLER, C.H. Inhibitory terpenes volatilized from Salvia shrubs. Bull. Torrey bot. Club 92, 1965: 38-45.

MULLER, C.H. The role of chemical inhibition (allelopathy) in vegetational composition. Bull. Torrey bot. Club 93, 1966: 332-351.

MULLER, C.H. and DEL MORAL, R. Soil toxicity induced by terpenes from Salvia leucophylla. Bull. Torrey bot. Club 93, 1966: 130-137.

MULLER, W.H. Volatile materials produced by Salvia leucophylla: effects on seedling growth and soil bacteria. Bot. Gaz. 126, 1965: 195-200.

MULLER, W.H. and MULLER, C.H. Volatile growth inhibitors produced by Salvia spp. Bull. Torrey bot. Club 91, 1964: 327-330.

NATIONAL ACADEMY OF SCIENCES. Biochemical interactions among plants. Washington D.C., Natl. Acad. Sciences, 1971, 134 p.

NEILSON-JONES, W. Biological aspects of soil fertility. J. agric. Sci., Camb. 31, 1941: 379-411.

PATRICK, Z.A. Peach replant problem II: Toxic substances from microbial decomposition products of peach root residues. Can. J. Bot. 33, 1955: 461-486.

PATRICK, Z.A. Phytotoxic substances associated with the decomposition in soil of plant residues. Soil Sci. 111, 1971: 13-18.

PATRICK, Z.A., TOUSSOUN, T.A. and KOCH, L.W. Effect of crop residue decomposition products on plant roots. A. Rev. Phytopath, 2, 1964: 267-292.

PERSIDSKY, D.J. and WILDE, S.A. Effect of volatile substances released by soil, humus and composts on the growth of excised roots. Pl. Physiol., Lancaster 29, 1954: 484-486.

PERSON, H.L. and HALLIN, W. Natural restocking of redwood cutover lands. J. For. 40, 1942: 683-688.

PLHAK, F. and URBANKOVA, V. Study of the effect of volatile substances from cereal roots. Biologia Pl. 11, 1969: 226-235.

RICE, E.L. Chemical warfare between plants. Bios 38, 1967: 67-74.

SAMTSEVICH, S.A. and BORISOVA, V.N. Data on the toxicity of volatile compounds produced by saprophytic soil micro-organisms. (Russ.) Mikrobiologiya 32, 1963: 484-491. (from Biol. Abstr. 44, 1963)

SCOTT, C.W. Pinus radiata. F.A.O. Forestry and Forest Products Study No. 14, 1960, 328 p.

SMITH, A.M. and COOK, R.J. Implications of ethylene production by bacteria for biological balance of soil. Nature, Lond, 252, 1974: 703-705.

SMITH, K.A. and DOWDELL, R.J. Gas chromatographic analysis of the soil atmosphere: Automatic analysis of gas samples for O_2, N_2, Ar, CO_2, N_2O and C_1-C_4 hydrocarbons. J. Chromat. Sci. 11, 1973: 655-658.

SMITH, K.A. and RESTALL, S.W.F. The occurrence of ethylene in anaerobic soil. J. Soil Sci. 22, 1971: 430-443.

SMITH, R.S. Decline of Fusarium oxysporum in the roots of Pinus lambertiana seedlings transplanted into forest soils. Phytopath. 57, 1967: 1265.

SOKAL, R.R. and ROHLF, F.J. Biometry. San Francisco, Freeman, 1969; p. 239.

STEWART, E.R. and FREEBAIRN, H.T. Ethylene, seed germination and epinasty. Pl. Physiol., Lancaster 44, 1969: 955-958.

STONE, E.L. and WILL, G.M. Nitrogen deficiency of second generation radiata pine in New Zealand. Paper presented at 2nd North American Forest Soils Conference, 1963. In Forest-soil relationships in North America. Chicago, Chicago Univ. Press, 1965, p. 117-139.

TITOV, Y.V. Effect of spruce roots on the biological activity and nitrogen regime of podsolic soils. (Russ.) Lesoved 4, 1971: 9-20. (from For. Abstr. 33, 1972)

TRAPPE, J.M. Strong hydrogen peroxide for sterilizing coats of tree seed and stimulating germination. J. For. 59, 1961: 828-829.

TUSTIN, J.R. and KNOWLES, R.L. Integrated farm forestry. N.Z. Jl. For. 20, 1975: 83-88.

WEBB, L.J., TRACEY, J.G. and HAYDOCK, K.P. A factor toxic to seedlings of the same species associated with living roots of the non-gregarious subtropical rain forest tree Grevillea robusta. J. Appl. Ecol. 4, 1967: 13-25.

WHYTE, A.G.D. Productivity of first and second crops of Pinus radiata on the Moutere gravel soils of Nelson. N.Z. Jl. For. 18, 1973: 87-103.

WILL, G.M., APPLETON,E.J., SLOW, L.J. and STONE, E.L. Boron deficiency - the cause of dieback of pines in the Nelson district. For. Res. Instit., N.Z., Leaflet No. 1, 1963, 1 p.

APPENDICES

1. Climatic and edaphic conditions at Ashley Forest, Canterbury.

Altitude: 107 m A.S.L.

Annual rainfall: 720 mm

Annual raindays: 111 days

Mean summer temperature: 17.1°C

Mean winter temperature: 7.8°C

Soil type: Makerikeri hill soil, a yellow grey earth; Makerikeri hill soils occur as "mostly stony silt loams with interbedded greywacke gravels, silts and clays with some ridges of loess mantle". The topography is "mostly steep with rolling ridge tops" and the nutrient status is low. (N.Z. Soil Bureau Bulletin No. 27, 1968)

2. Climatic and edaphic conditions at Eyrewell Forest, Canterbury.

Altitude: 158 m A.S.L.

Annual rainfall: 700-900 mm

Annual raindays: 100-165 days

Mean summer temperature: 18°C

Mean winter temperature: 6.3°C

Soil type: Lismore very stony silt loam, a yellow grey earth; Lismore soils are shallow, stony, very free draining and very droughty. They consist of a thin veneer of loess, 10cm to 40cm deep, over greywacke gravels, stones and boulders in a matrix of sand and silt on flat land. They have a low nutrient status. (N.Z. Soil Bureau Bulletin No. 14, 1967)

ADDENDA

I. MATERIALS AND METHODS

1. the lacquered interior of the containers prevented corrosion of the galvanised iron.

2. Litter samples were collected at intervals of three to seven weeks throughout the year and were used immediately. Moisture content varied between 23% and 66% of wet weight and was not adjusted. The litter cores were placed in the containers without packing and there was normally 3cm - 5cm air space above the litter.

3. In preliminary experiments, a litter sample (190g wet wt.) was sealed in a glass crystallisation container (23cm in diameter and 7cm deep) and incubated for 48 h at 25°C. An open Petri dish containing assay seedlings was then enclosed in the container of litter and the apparatus incubated for 48 h before measurement of the seedlings.

4. Assay jars and Seed Test papers were autoclaved (20 min at 138 kPa) before use and 10 ml sterile distilled water used to wet each paper. The metal lids had a rubber sealing washer which formed an airtight joint with the lip of the assay jars.

5. Although the boiling point of N_2 (-195°C) is lower than that of O_2 (-183°C), there were no problems caused by either back-pressure or condensation of O_2 in the trap. Control seedling growth in the assay jars treated with gas which had passed through the trap was not restricted, indicating the presence of sufficient O_2 in the jars for normal plant growth.

II. RESULTS

6. Collections of litter for clover hypocotyl growth assays were made over summer, autumn and winter. There was no relationship between the inhibitory activity of the litter vapour and the season.

Date of litter collection	Clover hypocotyl growth (% of control)
21st January	34.40
12th February	33.40
1st April	37.15
1st May	36.11
10th June	26.47
7th August	37.26

7. The efficiency of the $KMnO_4$ trap would be reduced as components of the gas stream were oxidised.

Reduction of control germination by the $KMnO_4$ trap (Table 15) could indicate the removal of a stimulant from the control vapour. It is probable that sufficient C_2H_4 is present in the controls to explain this effect.

8. The moisture content of the litter used in these experiments was measured but there was no correlation between moisture and the effect of litter vapour on clover germination.

Litter moisture (% wet wt.)	Clover germination (% control)
22.5	185.9
36.6	17.8
52.9	64.4
58.5	128.1
66.3	59.9

9. The efficiency of the $KMnO_4$ trap at removing C_2H_4 (5ppm) from a gas stream (0.8 litre min^{-1}) was ; 99%. The concentration of C_2H_4 in litter vapour was reduced by the trap to less than 0.03ppm (Table 13) indicating an efficiency of greater than 99%.

10. Jars of litter were heated in a water bath at 80°C for 30 min. The rate of increase of the internal air temperature is given below. The presence of moist litter in the jars would tend to increase the rate of temperature rise.

Time in 80°C water bath (min)	Internal air temperature (°C)
0	21
5	60
10	71
15	74
20	75
25	76
30	76

11. Apart from the disturbance treatment which took about one minute, disturbed and undisturbed litter were treated identically so that their moisture contents would not differ.

12. One litter core was taken for each jar to keep sampling disturbance to a minimum.

13. The litter used in this experiment had a moisture content of 53% when sampled and, because the 8 mm hole was the only opening during the "rest" period, drying of the litter was restricted.

14. Vapour was sampled at Ashley Forest on April 21st when the moisture content of the litter was 45% and at Eyrewell on May 1st when the moisture content was 66%.

III. DISCUSSION

15. Forty samples were taken in subsequent litter collections.

16. There was no correlation between seedling growth and the order in which the jars were treated with litter vapour.

17. Mercuric perchlorate is efficient at absorbing C_2H_4 and is frequently included in experimental vessels to reduce C_2H_4 concentrations to very low levels. Because the reaction is slow it is not suitable for scrubbing C_2H_4 from a gas stream (Abeles, 1973, p. 23) which explains why the trap was inefficient.

18. A feature of the pine litter was the presence of large wefts of fungal mycelium which bound the needles together into cohesive clumps. This mycelium, in all samples examined, possessed clamp connections and therefore could be identified as basidiomycetous. Attempts to culture the mycelium on a variety of nutrient agar media (P.D.A., malt extract, corn meal) were unsuccessful. These fungi could be growing in mycorrhizal association with the pine roots and have critical requirements for culture conditions.

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PRODUCTION OF ETHYLENE BY INCUBATED LITTER OF *PINUS RADIATA*

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(*Accepted* 7 *July* 1975)

Summary—The inhibitory effect of vapour from incubated *P. radiata* D. Don litter to clover (*Trifolium repens* L.) hypocotyl growth was completely removed by $KMnO_4$ and −195°C traps, but not affected by a cold trap at −100°C. Plumule growth of rice (*Oryza sativa* L.) seedlings was stimulated by litter vapour, and this stimulation was removed by the $KMnO_4$ trap. A peak with the same retention time as ethylene was detected in the litter vapour by gas chromatography. The addition of C_2H_4 to vapour which had been passed through the $KMnO_4$ trap fully restored inhibition.

INTRODUCTION

In pure stands of *Pinus radiata* D. Don in Canterbury Province, South Island, New Zealand, a heavy litter layer accumulates, and undergrowth is sparse or absent. Under laboratory conditions the litter has been shown to produce a volatile factor which inhibits the shoot growth of white clover, perennial ryegrass, and *P. radiata* seedlings (Lill and Waid, 1975). If this volatile factor accumulates in the field, it could be at least partially responsible for the sparse undergrowth in the *P. radiata* forests. The work described here was carried out to establish the identity of this volatile factor.

MATERIAL AND METHODS

Sampling and bioassay

The complete litter horizon under a mature *P. radiata* stand (39y, 300 stems/ha) at Ashley Forest, Canterbury, was sampled. Forty litter cores (10 cm dia) and a beaker containing 200 ml H_2O were placed in each 18 l incubation container. The containers were sealed and incubated at 25°C for 4 days after which the internal atmosphere was sampled for assay. In some experiments the containers were resealed and incubated for a further 7 days. Containers with no litter were treated identically as controls.

Grasslands 'Huia' white clover (*Trifolium repens* L.) and 'Tan-ginbozu' rice (*Oryza sativa* L.) seeds were surface sterilised with 1·4% Janola (NaOCl) for 5 min, rinsed with distilled H_2O, and germinated for 24 h (clover) or 60 h (rice). Fifteen clover or ten rice seedlings were placed in each assay jar, and vapour from the incubation containers passed over them (Fig. 1). When 1 l. of vapour had been flushed through each jar, the hole in the metal seal was plugged with a cork enveloped in polythene. The jars were incubated at 25°C for 4 days (clover) or 5 days (rice) before the hypocotyls or plumules were measured to the nearest 1 mm.

Treatment of vapour

Litter vapour was passed through various traps before assay to help classify the inhibitor. These traps were included between the incubation container and the manometer (Fig. 1).

A permanganate trap was used to indicate whether the inhibitor could be oxidised readily. Silica gel (100 g) treated with 125 ml 0·1 M $KMnO_4$ was dried overnight at 105°C and placed in a gas washing bottle.

A cold trap (Dal Nogare and Juvet, 1965) was used with two coolants: dry ice-diethyl ether (−100°C, The Merck Index, 1969) and liquid N_2 (−195°C). Litter vapour was first stripped of CO_2 and H_2O using 0·9 M NaOH and anhydrous $MgSO_4$ respectively.

Gas chromatography

Gas samples were chromatographed on a 1·75 m × 3·2 mm Cu column packed with 80–100 mesh Porapak-Q in a Tracor 550 G.C. using a flame ionisation detector. N_2 was used as the carrier gas at a flow rate of 60 ml min^{-1}, and a column oven temperature of 65°C. Because of poor separation of C_2H_4 and C_2H_2 on this column, a second column was used in the last experiment. This was a 3·05 m × 3·2 mm Cu column packed with alumina deactivated with NaI (Smith and Dowdell, 1973) with a N_2 flow rate of 30 ml min^{-1} and an oven temperature of 120°C.

Samples (0·5 ml) were injected with a 1 ml Hamilton gas tight syringe. Standards were dilutions of C_2H_2 or C_2H_4 supplied by N.Z. Industrial Gases Ltd. The mean peak height of three injections was used to calculate concentrations.

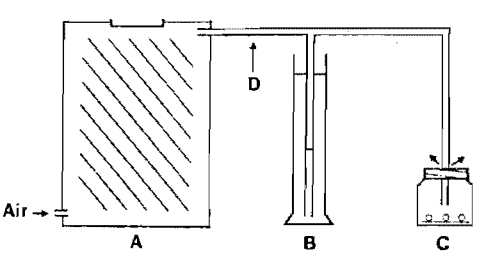

Fig. 1. Apparatus used to assay vapour from incubated litter. A, incubation container. B, water manometer. C, assay jar. D, position of traps.

Table 1. The inhibition of clover hypocotyl growth (back-transformed means in mm*) by vapour from incubated *P. radiata* litter and the effect of $KMnO_4$ and cold traps on this response

Time (days) of litter incubation	Container contents	Treatment of vapour			
		Untreated	$KMnO_4$ trap	$-195^{\circ}C$ trap	$-100^{\circ}C$ trap
4	Control	19.52^{ab}	20.34^{ab}	23.15^{a}	
	Litter 1	4.91^{def}	21.02^{ab}	18.86^{b}	
	2	5.05^{def}	21.46^{ab}	22.96^{a}	
	3	5.14^{def}	21.05^{ab}	20.15^{ab}	
11	Control	19.92^{ab}			18.20^{b}
	Litter 1	4.58^{ef}			4.47^{f}
	2	5.74^{d}			6.87^{c}
	3	5.44^{de}			5.31^{def}

Each mean is derived from 5 jars of 15 seedlings. Coefficient of variation of transformed data = 13%.

*Because of the heterogeneity of variance, log transformations of the data were used in the analysis. Means with the same superscript are not significantly different at the 1% level as determined by the Student-Newman-Keul test on the log transformed data.

RESULTS

Effect of $KMnO_4$ and cold traps

When litter vapour was passed through a $KMnO_4$ trap the inhibitory effect was removed (Table 1), indicating that the inhibitor is readily oxidised. The $-195^{\circ}C$ trap also removed the inhibition, while the $-100^{\circ}C$ trap had no effect. A compound with these characteristics and which might cause these inhibitory effects is C_2H_4. It is readily oxidised by $KMnO_4$, boils at -103 C, freezes at -181 C, and at low concentrations inhibits seedling growth (Abeles, 1973).

Effect on rice seedlings

It has been shown that seedling growth of some monocotyledons, e.g. rice (Ku *et al.*, 1970) is stimulated by C_2H_4 so the litter vapour was assayed using rice plumule growth. There was a significant stimulation of growth which was removed by passing the vapour through the $KMnO_4$ trap (Table 2).

Effect of ethylene

Preliminary analysis of the litter vapour on the Porapak-Q column indicated a peak with the same retention time as C_2H_4. C_2H_2 and C_2H_4 are not very well separated on this column, so the effect of these gases on clover seedlings was tested (Table 3). C_2H_2 had no effect on seedling growth while C_2H_4 caused inhibition.

Table 3. The effects of litter vapour, acetylene, and ethylene on clover hypocotyl growth (back-transformed means in mm*)

	Control	Litter vapour	Acetylene (9.00 parts/10^6)	Ethylene (9.93 parts/10^6)
Growth (mm)	21.34^{a}	7.76^{b}	20.65^{a}	4.75^{c}

Coefficient of variation of transformed data = 9%

*See Table 1.

A second column which effects a better separation of C_2H_4 from C_2H_2 was used in subsequent experiments to avoid possible confusion between these gases. With this column preliminary analysis indicated a concentration of C_2H_4 in the assay jars of *ca* 5 parts/10^6. Passing litter vapour through the $KMnO_4$ trap completely removed the C_2H_4 peak, and also reduced inhibition to the same level as the untreated control (Table 4). Addition of C_2H_4 to vapour which had passed through the $KMnO_4$ trap resulted in a complete restoration of the inhibitory effect. Gas analysis showed that C_2H_4 concentrations in treated litter vapour with C_2H_4 added and in untreated litter vapour were similar.

DISCUSSION

The evidence presented here indicates that during incubation of *P. radiata* litter samples C_2H_4 is produced in sufficient quantities to cause inhibition of clover hypocotyl growth and stimulation of rice plumule growth under the assay conditions used. Reports on the effects of C_2H_4 on dicotyledonous seedling growth (Abeles, 1973) suggest that inhibition of elongation is half maximum at 0·1 parts/10^6 and is maximal at 10 parts/10^6. C_2H_4 concentrations measured in vapour from incubated litter are high enough to have a near maximum effect. All the inhibition detected could be attributed to the C_2H_4 present in the vapour.

Although no conclusions can be drawn on field concentrations this work indicates that the potential for C_2H_4 production is present. If the source of C_2H_4 is microbial then production in the field is likely to

Table 2. The effect of vapour from incubated *P. radiata* litter on rice seedling growth (back transformed means in mm*)

Container contents	Untreated vapour, 4 days litter incub.	Untreated vapour, 11 days litter incub.	Vapour through $KMnO_4$ trap, 11 days incub.
Control	19.15^{d}	19.90^{d}	20.33^{d}
Litter 1	26.21^{bc}	29.34^{ab}	20.58^{d}
2	24.71^{c}	31.82^{a}	20.75^{d}
3	25.96^{bc}	32.78^{a}	20.03^{d}

Each mean is derived from 6 jars of 10 seedlings. Coefficient of variation of the transformed data = 9%.

*See Table 1.

Table 4. The effect on clover hypocotyl growth of litter vapour which had been passed through a $KMnO_4$ trap and the change in response due to the subsequent addition of ethylene to this vapour (back-transformed means in mm*)

Container		Treatment of vapour: Untreated		$KMnO_4$ trap		$KMnO_4$ trap + C_2H_4	
contents		Growth	C_2H_4 (Parts/10^6)	Growth	C_2H_4 (Parts/10^6)	Growth	C_2H_4 (Parts/10^6)
Control	1	19.81^{ab}		23.37^{a}	0**		
	2	20.00^{ab}				6.26^{c}	3.75
Litter	1	6.99^{c}	3.52	17.18^{b}	0**		
	2	6.34^{c}	5.06	18.68^{b}	0**		
	3	7.45^{c}	2.61			6.66^{c}	2.61
	4	5.88^{c}	7.44			6.76^{c}	3.98

Each mean is derived from 5 jars of 15 seedlings.

Coefficient of variation of the transformed data = 17%

*See Table 1

**Not detected at 0.01 parts/10^6.

increase with increasing summer temperatures. High concentrations of C_2H_4 in the litter layers in warm moist conditions would coincide with the germination of seeds present in the litter, and any delay in establishment of the seedlings at this stage would render them more vulnerable to the dry summer conditions which often prevail in Canterbury. On the other hand, the C_2H_4 may be produced by senescing plant tissue in the litter layer. Needles and twigs fall throughout the year and it is uncertain how long metabolic activity can persist after abscission. It would be difficult to distinguish C_2H_4 produced by senescing tissue from that produced by microorganisms.

Although the potential for C_2H_4 production may be present in the field, it is possible that actual production may occur only when the litter is mechanically disturbed. C_2H_4 is frequently produced in response to tissue damage (Abeles, 1973). Litter sampling involves cutting through plant tissue (feeder roots, needles and twigs, wefts of fungal mycelia, etc.) and this damage may be the cause of C_2H_4 production in the litter samples.

Further work will be carried out to determine whether litter disturbance is causing C_2H_4 production, and to what extent it is produced under normal field conditions.

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REFERENCES

Abeles F. B. (1973) *Ethylene in Plant Biology*. Academic Press, New York.

Dal Nogare S. and Juvet R. S. (1965) *Gas-liquid Chromatography*. Wiley, New York.

Ku H. S., Sugi H., Rappaport L. and Pratt H. K. (1970) Stimulation of rice coleoptile growth by ethylene. *Planta* **90**, 333–339.

Lill R. E. and Waid J. S. (1975) Volatile phytotoxic substances formed by litter of *Pinus radiata* N.Z. *J. For. Sci.* 00, 000–000.

The Merck Index (1969) p. 1310 8th edition, Merck, Rahway.

Smith K. A. and Dowdell R. J. (1973) Gas chromatographic analysis of the soil atmosphere: automatic analysis of gas samples for O_2, N_2, Ar, CO_2, N_2O and C_1—C_4 hydrocarbons. *J. Chromat. Sci.* **11**, 655–658.

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VOLATILE PHYTOTOXIC SUBSTANCES FORMED BY LITTER OF *PINUS RADIATA*

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ABSTRACT

Volatiles from radiata forest litter were shown to inhibit seedling growth of **Trifolium repens** L. (white clover), **Lolium perenne** L. (perennial ryegrass), and **Pinus radiata** D. Don. Seed germination of ryegrass and radiata was also reduced. This effect was not due to CO_2, reduced O_2 concentrations, or a compound soluble in water or paraffin wax. The authors have not yet shown that this effect occurs in the field.

INTRODUCTION

Pinus radiata D. Don is important economically in New Zealand not only as a forest crop but also when grown in association with agriculture. Under pure stands of radiata in Canterbury a thick layer of litter accumulates and the growth of seedlings of radiata and other species is sparse or absent. Because the lack of undergrowth might in part be due to allelopathy it was thought worthwhile to investigate the possible effects of radiata litter on the growth of seedlings of radiata and other plants.

There have been reports of the production of volatile metabolites by fungal cultures (Hutchinson, 1973), and because radiata litter supports vigorous fungal growth the effect of volatile substances evolved from the litter on seed germination and seedling growth was investigated.

MATERIALS AND METHODS

Basic Experimental Procedure

Litter was collected from beneath a mature stand of *Pinus radiata* (39-yr old, 300 stems/ha) at Ashley Forest, Canterbury. Despite moist site conditions undergrowth was sparse, and there were very few radiata seedlings even though numerous seed wings were seen in the litter.

In the first set of experiments cores (14 cm diam.) of the litter horizon, which ranged from 1.0 to 14.8 cm deep, were removed from positions determined by random

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co-ordinates from within the sampling area (100 m^2). Twenty litter cores were placed in 18-litre tins fitted with inlet and outlet tubes (Fig. 1). In later experiments 40 cores of litter cut with a 10 cm diam. steel corer were placed in each tin.

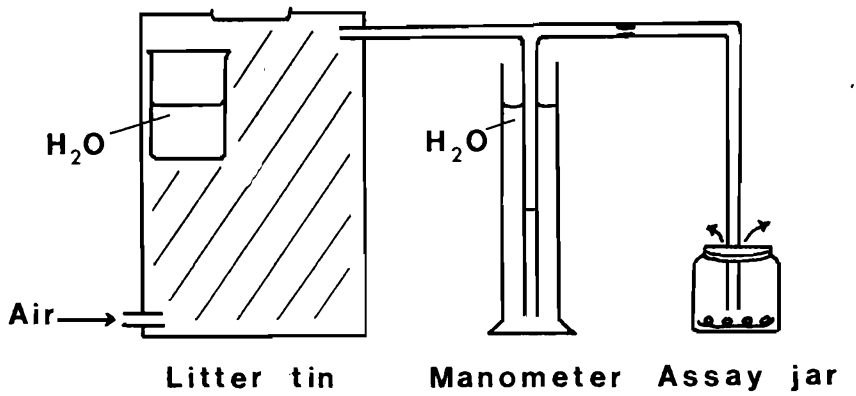

FIG. 1—Apparatus used for assaying litter volatiles

After placing a beaker containing 200 ml H_20 in each tin, the tins were sealed and incubated at 25°C for 4 days before the tin atmosphere was assayed. Control tins containing no litter were treated identically.

Preserving jars (500 ml "Agee" Utility) with a hole (8 mm diam.) in the metal seal were used as assay jars. Seedlings or seeds were placed on damp Whatmans seed test paper in each jar. Litter vapour for assay was displaced from the litter tins by compressed air and conducted by glass tubing into the assay jars (Fig. 1). A manometer was used to regulate the flow rate so that 1 litre of the litter atmosphere was passed into each assay jar. A loose fit of the glass tube in the lid of the assay jar permitted flushing of the jar. After treatment the holes were sealed with corks covered with polythene film. The jars were incubated in the dark at 25°C.

For seedling assays white clover and perennial ryegrass seed (supplied by Grasslands Division, D.S.I.R., Lincoln) were surface sterilised in 14% (v/v) "Janola" (NaOCl) for 5 min, rinsed with sterile distilled H_2O and germinated on moist sterile filter paper in the dark at 25°C. Radiata seed (supplied by Forest Research Institute, Rotorua) was washed in running tap water for 24 hours, stratified at 3°C for 24 hours, surface sterilised in 100 vol. H_2O_2 for 20 min., and germinated in sterile vermiculite at 25°C. Day-old clover, 2-days-old ryegrass and 5-days-old radiata seedlings were selected for assay. The assay jars were incubated for 4 days (clover and ryegrass) or 7 days (radiata). After incubation the hypocotyl length of clover and radiata seedlings, and the plumule length of ryegrass seedlings (combined length of the mesocotyl, coleoptile, and the first leaf) were measured to the nearest 1 mm.

Conditions for the seed germination tests, in which the same seed stocks were used, are given in section (b) below.

Using the basic procedures described above the following were investigated:

(a) *Effects of litter volatiles on seedling growth*

Volatiles in the atmospheres of six tins of litter (20 cores/tin) and two control tins

after 4 days' incubation were assayed with clover and ryegrass seedlings. Five jars of seedlings of each species per tin were treated consecutively. The litter volatiles were reassayed with clover after a further 7 days' incubation.

With a second collection of litter (20 cores/tin) one group of three tins with litter and one control tin was assayed with clover and ryegrass seedlings, and a second group with clover and radiata.

(b) *Effects of litter volatiles on seed germination*

Using the first collection of litter the effect of litter volatiles on the germination of surface-sterile seed of ryegrass and clover was investigated. Assay jars containing 300 seeds of ryegrass or clover were treated with vapour. Each tin was assayed with one jar of clover and another of ryegrass so that for each species there were two control and six experimental jars. Germination percentages were determined after 3 and 4 days' incubation at 25°C for clover and ryegrass respectively. A seed was classed as germinated if the radicle protruded through the seed coat.

An assay using the same procedures was made on the second collection of litter but using ryegrass (2 jars with 200 seeds/tin) and radiata (3 jars of 100 seeds/tin). Radiata germination was measured after 7 days' incubation.

(c) *CO_2 accumulation and O_2 depletion*

Litter vapour was assayed with white clover seedlings after CO_2 had been removed by passing the vapour through a gas washing bottle containing 125 ml 0.9 M NaOH placed in the outlet tubing assembly from the litter container between the tin and the manometer (see Fig. 1). Two tins, each containing 40 cores of litter, and one control tin were assayed with the CO_2 trap in place with 5 jars of white clover seedlings. The assay was repeated without the CO_2 trap. The NaOH was titrated with 1.25 M HCl to measure the CO_2 trapped from each tin.

Vapour obtained from a second group of two tins of litter and one control tin was replenished with O_2 to reduce or eliminate any effects of O_2 depletion by decomposing litter in the sealed tins on the growth of white clover seedlings in the assay jars. Any CO_2 formed in the tins was trapped in 200 ml 1.5 M NaOH held in beakers, replacing those containing water. Ten assay jars were treated per tin. Into five of these 50 ml O_2 was injected by hypodermic syringe to give a possible maximum and minimum of 28 and 10% respectively. Fifty ml N_2 was injected into each of the other five jars to act as controls. These jars could have contained a maximum and a minimum of 18 and 0% O_2 respectively. It was thus possible to detect (in the absence of CO_2) the effects of any O_2 deficiency.

(d) *Influence of paraffin wax and water traps on inhibitory effects of litter volatiles*

As monoterpenes have been shown to be involved in allelopathy (Muller, 1966) litter vapour was assayed after it had been passed over paraffin wax to trap any monoterpenes present. A gas washing bottle was packed with paraffin wax shavings and included in the gas line between the tin and the manometer (see Fig. 1).

To check the effectiveness of the trap, a small amount of camphor was placed in a

5-litre flask and left overnight to evaporate. When this vapour was passed through the trap three observers could not detect the characteristic odour of camphor though the smell was strong if the trap was removed.

One control and two litter tins (40 litter cores/tin) were assayed using white clover seedlings. Each tin was assayed with five jars with vapour which had passed through the trap and five with untreated vapour.

Because some volatile primary metabolites are soluble in water, e.g., ethanol, acetic acid and acetone, litter vapour from a second group of containers was assayed after passing through a gas washing bottle containing 125 ml H_2O.

RESULTS

(a) *Seedling growth*

In the experiments with the first two collections of litter, the presence of volatiles evolved from radiata litter was associated with a significant reduction in seedling growth (Table 1). With the first collection after 4 days' incubation there was significant variation in the inhibitory effect of litter volatiles from different containers on the growth of ryegrass and clover seedlings but such variation was not evident after 11 days' incubation in both the control and the litter vapour treatment. However, there was little change in the level of inhibition, which was 36.3% and 39.0% of control respectively for 4 and 11 days of litter incubation.

TABLE 1—The effect of vapour from incubated **P. radiata** litter on the growth of ryegrass, white clover and radiata seedlings (back-transformed means in mm*)

Litter collection	Time (days) of litter incubation	Species	Treatment		C.V. of transformed data
			Control	Litter vapour	
1st	4	Ryegrass	27.76[a]	20.58[b]	
	4	Clover	17.61[c]	6.40[e]	
	11	Clover	15.24[d]	5.94[f]	23%
2nd	4	Ryegrass	32.05[g]	21.71[h]	
	4	Clover	18.18[h]	5.86[j]	
	4	Radiata	21.96[h]	13.36[i]	28%

* Because of heterogeneity of variance, data was transformed using the log transformation before computing a nested analysis of variance. Group means of the transformed data were compared using the Student-Newman-Keul's test, and the results of this test are given in the table of back-transformed means. Means not significantly different at the 5% level have the same superscript. Each litter collection was analysed separately and no comparisons have been made between collections.

(b) *Seed germination*

With the first collection of litter (Table 2) the proportion of white clover seed that germinated was increased by litter volatiles. However, the volatiles reduced germination of both ryegrass and radiata seed. These effects probably indicate changes in germination rate rather than total germination.

TABLE 2—The effects of vapour from incubated **P. radiata** litter on the germination % of clover, ryegrass and radiata seed

Litter Collection	Time (days) of litter incubation	Species	Treatment		Probability of a greater X^2 value
			Control	Litter vapour	
1st	4	Clover	79.9	84.7	0.01
	4	Ryegrass	77.0	59.2	<0.001
2nd	11	Ryegrass	81.8	42.3	<0.001
	11	Radiata	40.6	16.1	<0.001

(c) *CO_2 and O_2 effects*

Litter volatiles adversely affected white clover seedling growth both in the presence (5.5%) and the absence of CO_2 (Table 3). Rather more inhibition occurred where CO_2 had been removed from the vapour, and it is possible that substances antagonistic to the inhibitor were removed in the trap.

TABLE 3—The response of clover hypocotyl growth to litter vapour after removal of CO_2, and water and wax soluble substances and after addition of O_2 (back-transformed means in mm*). Litter incubation time was 4 days

Litter collection	Treatment of vapour	Treatment		C.V. of transformed data
		Control	Litter vapour	
3rd	untreated	19.26[a]	7.52[b]	
	NaOH trap	19.52[a]	5.51[c]	22%
4th	50 ml O_2/jar	19.00[d]	5.92[e]	
	50 ml N_2/jar	20.63[d]	6.53[e]	14%
5th	untreated	19.04[f]	7.14[g]	
	water trap	18.44[f]	6.47[h]	
	wax trap	19.27[f]	6.55[h]	18%

* See Table 1.

(d) *Influence of paraffin wax and water traps*

The presence of these traps slightly increased the inhibitory effect of litter volatiles (Table 3). It may be that, as suggested in section (c) above, substances antagonistic to the inhibitor are removed in these traps. However, it is evident that the inhibitor is not absorbed effectively in either water or wax traps.

DISCUSSION

The experiments described above have demonstrated the presence of a volatile substance (or a mixture of volatile substances) evolved from incubated radiata litter that inhibits the growth of radiata, ryegrass and white clover seedlings and the germination of radiata and ryegrass seed, and stimulates the germination of clover seeds. The active substance has not been identified but it does not appear to be CO_2, a water-soluble metabolite, or a monoterpene. The experiments have not demonstrated whether the inhibitory volatile is evolved from *Pinus radiata* tissues *per se,* or if formed during their decomposition by litter-inhabiting organisms.

It is possible that the effects recorded in the laboratory result from substances produced in response to the mechanical disturbances of the litter during sampling, either through the release of additional substrates for microbial activity, or from a specific physiological reaction to damage.

We are endeavouring to identify the volatile compound and to determine if it occurs in significant quantities under field conditions.

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REFERENCES

HUTCHINSON, S. A. 1973: Biological activities of volatile fungal metabolites. **Ann. Rev. Phytopath. 11:** 223-46.

MULLER, C. H. 1966: The role of chemical inhibition (allelopathy) in vegetational composition. **Bull. Torrey bot. Cl. 93:** 332-51.